Klinik für Reproduktionsmedizin
Abteilung für Grosstierreproduktion
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. med. vet. Dipl. ECBHM Heiner Bollwein

Arbeit unter wissenschaftlicher Betreuung von
Dr. Cameron Moshfegh

Investigation of the tumorigenicity of GoST cells derived from miPS cells

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Sebastian Giovanni Rambow

Tierarzt
aus Bergfeld, Neustrelitz, Deutschland

genehmigt auf Antrag von

Prof. Dr. med. vet. Dipl. ECBHM Ulrich Bleul, Referent
Prof. Dr. Dr. h. c. Viola Vogel, Korreferentin

2017

Table of Contents

Abstract

Pluripotent stem (PS) cells retain the ability to differentiate into cells representing the three germ layers, mesoderm, ectoderm and endoderm. Embryonic stem (ES) cells isolated from blastocysts and reprogrammed somatic cells, described as induced pluripotent stem (iPS) cells, are a promising source of PS cells for regenerative medicine. PS cells hold the potential to cure cell-specific diseases through autologous transplantation of cells or tissues by prior differentiation into specific cell types. If PS cells are transplanted into an immunocompatible host they form multidifferentiated tumors, described as teratomas. The control over the differentiation process of PS cells still remains a challenge. Reducing or eliminating the intrinsic tumorigenicity of PS cells may lead to safer stem cell therapies for treatments of yet incurable diseases. A recently developed technology induces early germ cell features in male ES cells, defined as gonogenic stimulated transition (GoST) induction. The resulting cells are described as GoST cells. In this work we investigated if GoST induction is possible in male murine iPS (miPS) cells and tested the tumorigenicity of miPS-derived GoST cells in the NSG mouse model. Although GoST cells still contributed to teratoma formation, our results showed reduced tumor growth of GoST cells compared to wild type (WT) miPS cells. By extending an unstimulated time period after GoST induction, expression of spermatogonial stem cell markers was observed.

Zusammenfassung

Pluripotente Stammzellen (PS Zellen) sind fähig sich in Zellen zu differenzieren, die die drei Schichten des Urkeims repräsentieren. Embryonale Stammzellen (ES Zellen) aus Blastozysten und reprogramierten somatischen Zellen, sogenannte induzierte pluripotente Stammzellen (iPS Zellen), sind eine vielversprechende Quelle von PS Zellen für die regenerative Medizin. PS Zellen haben das Potential, zellspezifische Krankheiten durch autogenen Ersatz von Zellen oder Geweben durch Differenzierung in den jeweiligen Zelltyp zu ermöglichen. Autogen transplantierte PS Zellen können jedoch multidifferenzierte Tumore bilden, sogenannte Teratome. Die Reduktion oder Eliminierung der Tumorigenität würde eine sichere Anwendung von Stammzellen bedeuten. Eine neu entwickelte Technik induziert frühe Keimzelleigenschaften in ES Zellen, die sogenannte gonogenic stimulated transition (GoST) induction. Die daraus entstandenen Zellen werden als GoST Zellen bezeichnet. In dieser Arbeit wurde die Möglichkeit der „GoST induction“ in murinen iPS Zellen und die

Tumorigenität der GoST Zellen im NSG Mausmodell untersucht. Obwohl GoST Zellen zur Teratombildung beitrugen, zeigten unsere Ergebnisse ein reduziertes Wachstum im Vergleich zu Wildtyp miPS Zellen. Durch Verlängerung der unstimulierten Kulturzeit von GoST Zellen nach der „GoST induction“ wurde die Expression von spermatogoniellen Stammzellmarkern beobachtet.

1. Scope of Thesis

1.1 Motivation

In contrast to somatic stem cells, which are found in differentiated tissues and can only differentiate into specialized cell types of the tissue or organ, PS cells can differentiate into cells of all three germ layers. PS cells can be isolated and propagated indefinitely when cultured *in vitro* in contrast to somatic stem cells. The first PS cells of interest were embryonic carcinoma cells, but weren't applicable in medicine due to their malign background. Human ES (hES) cells were the first PS cells considered as a cellular source for stem cell therapy in regenerative medicine, but were associated with several problems. These include ethical issues related to embryo destruction and immunocompatibility between donor and host. Autologous iPS cells are now considered to be a more reliable source of PS cells for therapeutic applications. iPS cells circumvent the immunological and ethical disadvantages of ES cells. Since iPS cells can be obtained by and re-transplanted into the same patient they share an identical genetic background and embryonic death can be avoided. The concept behind stem cell therapy is the differentiation of PS cells into progenitor cells prior to transplantation. The control over the differentiation process remains one of the most challenging tasks in stem cell therapy. Undifferentiated PS cells lead to multidifferentiated tumors when transplanted into an immunocompatible host. This type of tumor can be benign in form of a teratoma or malignant in form of a teratocarcinoma. But either way, tumor growth must be avoided at any point of a stem cell therapy. Different approaches to gain control over the differentiation process of PS cells have already been made. For example by introducing a suicide gene for PS cells in the genome, selecting differentiated cells via chemicals toxic for PS cells or cell sorting for undifferentiated cells. None of these techniques was able to reliably eliminate PS cells from differentiated cell populations until now. Gaining reliable and precise control over the differentiation process would lead to safer therapies involving stem cells and improve the understanding of the regulatory processes during differentiation of PS cells.

In this context we investigated the tumorigenicity of a recently developed technology to induce early germ cell features in PS cells, the gonogenic stimulated transition (GoST) induction. The resulting cells are described as GoST cells. These cells hold the potential to

restore fertility in sterile men e.g. after chemotherapy or hereditary infertility. They also have the potential to be used for generating genetically modified organisms (GMO's) such as mice or livestock. This might improve animal welfare in science and simplify the generation of GMO's in contrast to classical methods such as embryo transfer or *in vitro* fertilization. We therefore adapted the GoST induction to culture conditions of murine iPS (miPS) cells with additional inhibiting factors in the culturing system.

1.2 Objectives

1.2.1 Objective 1

The first objective was to translate the GoST induction from mES cells to miPS cells and characterize the pluripotency of an isolated miPS single cell clone compared to mES cells. Comparing the pluripotency of mES and miPS cells represents a necessary step if iPS cells are supposed to replace ES cells in regenerative medicine. Pluripotency includes the ability to differentiate into cell types of the three germ layers. Since iPS cells would circumvent host rejection as well as ethical issues, they are the most promising alternative to ES cells as a PS cell source for regenerative medicine. To apply PS cells in regenerative medicine it is of major importance to gain knowledge about migration and development of transplanted cells in the host. Previously, miPS cells were lentivirally transduced with the two reporter transgenes luciferase and the lactose operon LacZ. A single cell clone with high expression of reporter transgenes was selected for optimal detection of the cells *in vivo.* This made it possible to track and distinguish the transplanted cells from somatic cells *in vivo*. Evaluating differences between mES and miPS cells represents an important step for future experiments. Differences may influence experimental results and future miPS cell applications.

In a previous work using mES cells, GoST cells showed enhanced multilineage differentiation potential and expressed markers of early male germ cells[1]. GoST cells may have potential as a stem cell source for regenerative medicine including fertility restoration in the male. Since pluripotency is correlated with tumor growth, the tumorigenicity of miPS-derived GoST cells had to be assessed. Another issue is the genetic stability of the cells during culture. Since genetic alterations of transplanted cells can harm either the patient or the offspring if introduced into the germline, karyotyping was performed, which however only can detect chromosomal abnormalities. Other analyses necessary to assess integrity of the genome, such as sequencing, were not performed here.

1.2.2 Objective 2

Another objective was further characterization of the GoST induction. GoST induction represents a chemical treatment which showed the potential to generate gonocyte-like cells from PS cells with enhanced multilineage differentiation pathways[1]. GoST cells survive in culture conditions deprived of the antioxidant β-mercaptoethanol (βME) and leukemia inhibiting factor (LIF), which are prerequisite ingredients in PS cell culture systems. GoST cells show reduced cell proliferation rates and experience a G1/S phase arrest and express core pluripotency markers[1]. Unraveling further cellular mechanisms represents an important step of understanding GoST induction. The GoST induction had been applied to mES cells but not to miPS cells. Therefore the effect of the GoST induction on miPS cells had to be assessed. The use of new culture conditions with addition of the Sirtuin1 and DNA methyltransferase (DNMT) inhibitors raised the question if GoST induction can be translated to miPS cells. Thus the comparison of GoST induced miPS cells with non-GoST induced miPS (WT) cells was necessary to gain further knowledge about the differentiation-state of miPS-derived GoST cells. In this context we used several *in vitro* techniques including real-time RT-PCR of mRNA and DNA as well as immunostaining and Western blot of pluripotency markers.

1.2.3 Objective 3

Introducing GoST cell-derived tissue or cells into the human body has to be safe. PS cells bear the risk of teratoma formation when transplanted into an immunocompatible host. GoST cells express pluripotency markers *in vitro.* Answering the question if GoST cells contribute to teratoma formation *in vivo* is an important step for future applications. Tumor growth during stem cell therapies embodies a major risk and prevents using PS cells therapeutically[2]. Therefore, we tested the tumorigenicity of GoST cells after seven days of GoST induction (D7) in comparison to WT miPS cells as positive control for PS cells. We chose the NSG mouse model for our *in vivo* experiments. NSG mice are amongst the most immunodeficient mouse strains to date and thus represent an ideal model for the transplantations of cells or tissues. This was a crucial part of this work, since GoST cells may help to restore fertility in the male through testis injections. A non-tumorigenic cell population is a prerequisite for any treatment including PS cells.

2. Introduction

2.1 Embryonic stem cells

Embryonic stem cells derived from the inner cell mass of blastocysts[3,4]. *In vitro* they proliferate indefinitely while retaining pluripotency to differentiate into the three germ layers. Pluripotency is defined by three main features: self-renewal, the ability to differentiate into all three germ layers and contribution to chimera formation when injected into a blastocyst[5]. When transplanted to an immunocompatible host *in vivo,* PS cells contribute to multidifferentiated tumors, described as teratoma. Teratoma formation is considered as an additional feature of PS and ES cells *in vivo* and is therefore used as an *in vivo* test for pluripotency[6,7]. The so called core pluripotency markers play an essential role for the self-renewal of PS cells. NANOG, Oct4 and Sox2[8-12] are transcriptional factors, which mediate pluripotency. They code for the essential proteins that maintain pluripotency in PS cells. NANOG is a transcription factor that is crucial to maintain self-renewal[13] and regulates the expression of other proteins involved in differentiation[14,15]. Oct4 and Sox2 form a binary complex to regulate the expression of certain genes[15]. Expression of pluripotency factors including *NANOG*, *Oct4* and *Sox2* is known to be linked to cancer[16]. Other factors like *Klf4* and *c-myc* also enhance the expression of core pluripotency markers[17,18] but are known oncogenes as well[19,20]. Core pluripotency markers and their enhancers are not solely regulating pluripotency. The primed state of pluripotency represents the state of the post-implantation epiblast[21,22] and cells show different expression patterns of cellular markers in comparison to naïve pluripotency[22]. Primed PS cells are destined towards certain cell lineages and have limited abilities to contribute to a whole embryo. In contrast to murine ES (mES) cells which represent the naïve state of pluripotency, human ES (hES) cells, when cultured *in vitro,* enter the primed state of pluripotency unless the state of naïve pluripotency is genetically or chemically stabilized[22]. *In vivo,* hES cells correspond to the native state of pluripotency as cells of the inner cell mass of the blastocyst. Efforts have been made to reset human ES cell to the naïve state of pluripotency by serum-free cell culturing techniques *in vitro*[23].

2.2 Induced pluripotent stem cells

Induced pluripotent stem (iPS) cells represent a great breakthrough in stem cell research. They were first derived from adult fibroblasts by reintroducing and overexpressing the so called Yamanaka factors *Oct4*, *Sox2*, *Klf4* and *c-Myc* (OSKM) via retroviral gene transfer into the genome[24,25]. They then expressed *Nanog*[26], *Oct4*[27], *Sox2* and *Klf4*[28] similar to ES cells and showed similar growth characteristics[25]. The potential to differentiate into multiple cell lineages[29-31] makes iPS cells a suitable replacement in stem cell research for ES cells. Although they share many characteristics with ES cells, they still differ in gene expression, DNA methylation[32] and chromatin modification[33]. They circumvent ethical and immunological reactions compared to embryonic stem cells, since autologous transplantation of reprogrammed somatic cells avoids immunological responses. These cells can provide a reliable foundation for pharmaceutical research and clinical applications in humans.

2.3 Germ cells

Germ cells give rise to the gametes during the development of an organism. Primordial germ cells (PGC's) are the first cells of the germ line[34]. PGC's migrate through the embryo from the hindgut, over the dorsal cord to their final destination in the emerging gonads, which later develop into the testis in males and ovaries in females[34-37]. Germ cells express specific markers like *stella* (also *Dppa3*)[38], *Prdm14*[38], *Cxcr4*[39], *Tex101*[40,41], *TRA98*[42], *Nanos2/3*[43,44] or *Dazl*[45] but also the pluripotency marker *Oct4*[46]. A recent discovery showed the potential of the pluripotency factor Nanog to induce germ cell differentiation in blastocysts[47], which indicates together with the *Oct4* expression that germ cells share markers with PS cells. Tex101 and TRA98 are specific markers for germ cells in males. Tex101 appears on the cell membrane of prospermatogonia in immature seminiferous tubes of the fetal testis during gonadal development[40,48] and plays an essential role in male fertility[49]. TRA98 is especially expressed in neonatal testis[42]. *Blimp1*, a zinc finger protein and transcriptional repressor gene, is another important marker of germ cells[50] and hematopoietic stem cells[51]. PS and germ cells share similarities in expression patterns besides *Oct4*[52,53]. Another important cellular feature of germ cells represents the demethylation of the genome[50]. Germ cells show a high degree of demethylation during their migration through the embryo, which enables genetic imprinting in the genome[54-57]. They finally migrate to the genital ridge and differentiate further into gonocytes and later into spermatogonia[46].

2.4 Spermatogonial stem cells

Spermatogenesis is maintained by spermatogonial stem cells (SSC's). SSC's either self-renew into SSC's or commit to differentiation into spermatozoa[58,59]. They express *Nanos2*[60,61], promyeloic leukemia zinc finger (*Plzf*, also *Zbtb16*)[62], *GDNF*[59] and *Gfra1*[63]. When cultured *in vitro* they grow as round cell colonies[64,65]. Gfra1 is a GDNF family receptor expressed in SSC's[63]. Nanos2 is a unique protein only expressed in germ cells in the sexually determined male gonad[66]. It promotes the male germ cell differentiation while suppressing the female program simultaneously[43]. Repression of differentiation[67,68] and meiosis[43] through Nanos 2 is essential for maintenance of SSC's[61]. Plzf is a zinc finger protein expressed throughout the embryogenesis, but also in the postnatal testis[62]. It controls the expression of lineage-specific target genes and thereby instructing cellular differentiation. Plzf knockout mice showed limited numbers of normal spermatozoa[62,69]. Damaged or dysfunctional SSC's can lead to permanent infertility in men and often occurs after chemotherapy[70,71]. Restoring fertility is one of the major focuses of human reproductive medicine[72].

2.5 GoST cells

A recent study investigated the effects of a chemical treatment to mES cells with the aim to introduce a proliferation arrest in absence of LIF and β-mercaptoethanol (βME) without the loss of cell viability. Usually deprivation from LIF and βME results in massive cell death of ES cells[73]. This culturing technology was described as gonogenic stimulated transition (GoST) induction[1]. The resulting cells were described as GoST cells. GoST induction includes a two-step chemical treatment. In a first step ES cells were conditioned by culturing ES cells in presence of the DNA methyltransferase (DNMT) inhibitor RG108 and the Sirtuin 1 (Srt1) inhibitor EX527 while LIF and βME were still present for 10 passages[1]. This step aimed at mimicking the genome wide demethylation in PGC's[74]. Resulting cells were described as conditioned ES (cES) cells. In the second step, the GoST induction, LIF and βME were removed from the medium and cES cells were cultured for seven days in presence of RG108, EX527 and tert-butylhydroquinone (tBHQ). tBHQ is an electrophilic redox-cycling compound which induces Nrf2-dependent antioxidant responses[75,76] and increasing intracellular reactive oxygen species (ROS) by undergoing redox cycling reactions[77]. Characterization of GoST cells revealed retained expression of the pluripotency markers *Oct4*, *Nanog* and *Sox2*[1]. However, GoST cells reportedly expressed the germ cell related markers *Nanos2, Tex101* and *TRA98*[1]. Furthermore GoST cells showed enhanced cardiogenic

and hepatogenic differentiation[1]. Additionally *Dax1*, a transcriptional repressor of multilineage differentiation pathways[78], showed downregulation in GoST cells during differentiation which has not been present in mES cells[1]. Thus, the question has been raised if GoST cells represent non-tumorigenic pluripotent stem cells for regenerative medicine. Approaches to eliminate tumorigenicity of PS cells usually interfere with the genome such as introducing a suicide gene for PS cells after differentiating into progenitor cells or tissues[79,80]. Alternatively, the potential of chemotherapy to eliminate PS cells after differentiation has been investigated[81]. Unlike these approaches the GoST induction aims at eliminating tumorigenicity in PS cells prior to differentiation. This distinct difference would make GoST induction a superior method for the generation of PS cells for regenerative medicine. The pluripotency of GoST cells together with germ cell-like features would make GoST cells an interesting cell source for clinical stem cell applications in reproductive medicine. Although GoST cells have been studied *in vitro*, the tumor formation potential remained unclear. As mentioned above, iPS cells can be a replacement for ES cells. Since GoST induction had been developed in murine ES cells and iPS cells require slightly different culture conditions, the question, if GoST induction can be performed on iPS cells was raised.

2.6 Stem cells in regenerative medicine

Since the discovery of PS cells from teratocarcinoma[82] and the first cultivation of murine ES cells[83], many approaches were made to develop stem cell therapies. The first successful stem cell therapy in humans was a bone marrow transplantation to cure a severe immunodeficiency[84]. Back then the doctors were not aware that they are transplanting hematopoietic stem cells[85]. With improving knowledge about stem cells and the recent discovery of creating iPS cells, treatments of yet incurable degenerative diseases might be possible[86]. In particular iPS cells would provide the best individual therapy for the patients. Especially in the field of neuroscience much effort is put into restoring the function of damaged or degenerated nerves e.g. in Huntington disease[87], Alzheimer's disease[88,89] and Parkinson[90] or treating brain cancer via stem cell therapy[91,92]. Tissues and organs with limited regenerative capacity such as muscle cells[30,93,94], the eye[95] and cartilage[96] represent further fields addressed by applied stem cell therapy. In the liver, stem cell therapy could provide a valuable tool for treating cirrhosis, an irreversible destruction of liver tissue[97]. Recent discoveries suggested the possible recovery of fertility of sterile men using stem cell based approaches[98-101]. Stem cell therapies have shown promising results in animal models[88,102] but

failed to translate into clinical trials to this day. The reasons range from legal and ethical issues regarding stem cell therapies in humans[103] to serious immunological reactions such as the graft-versus-host-disease, which provokes host rejection even between identical twins[104]. Tumor formation is another concern regarding clinical trials of stem cell therapies[105-108]. For example the formation of teratomas, a multi differentiated tumor consisting of all three germ layers, represents a major risk for the patient when using PS cells *in vivo*[2,107]. Autologous transplantation of iPS cells circumvents many of the ethical and immunological issues of stem cell therapies including ES cells. With the recent discovery of the powerful gene editing tool CRISPR/Cas9[109] the effectiveness of stem cell therapies can be raised. Genes edited with CRISPR/Cas9 could be tailored to each individual disease[110]. There have already been approaches to cure genetic diseases via CRISPR/Cas9 gene editing[111]. But again, those studies are so far only applicable *in vitro*[111] or in animal models[112] and have yet failed to progress into clinical studies for said reasons.

Eliminating possible side effects of stem cell therapy is not the only challenge for clinical trials. For example there can be alterations in the chromosome set due to culturing and processing of stem cells prior to application[113], which would lead to unpredictable side effects for the patient. This is especially dangerous if stem cells would be used in reproductive medicine, since damaged genomic DNA bares high risk for genetic diseases for further generations. Migration of stem cells should be avoided when injected to prevent metastasis in terminal vessel systems like lung, liver or kidney. Therefore, safe applications for stem cells in humans are not present until this day.

Since stem cell therapies work well in animal models, the possibility of creating transgenic animals via testis injection is possible[114].

2.7 Teratoma

Teratomas are benign tumors that contain differentiated cells from all three germ layers[115-117]. They are characterized by their rapid, mostly benign growth and heterogeneous mixture of tissues, such as hair, teeth, cartilage or skin[118]. Teratomas can transform into a malignant form, described as teratocarcinoma[116] which then contains embryonic carcinoma cells[117]. Teratomas can occur spontaneously *in vivo* e.g. in testis[119,120] or rarely in the spinal cord[121,122] or ovary[123]. When PS cells are transplanted into an immunocompatible host teratomas can

derive from PS cells[105,106]. Teratomas tend to not infiltrate surrounding tissue but can form metastasis in rare cases[124,125].

Since PS cells contribute to teratoma formation, the teratoma assay is used to evaluate the pluripotency of cells. Teratoma formation is declared as one of the key features of pluripotent cells[118,126]. Immunocompromised mouse models such as NSG or Nu/Nu represent ideal hosts for transplantation experiments and are therefore widely used for teratoma formation assays[6]. Teratoma formation does not only occur in the same species environment, but in cross-species transplantation as well[127].

Thus, teratoma formation is not only a valuable tool to evaluate the so called “stemness” of cells, meaning the pluripotency, but also bears a great challenge for applied stem cell therapy in humans.

3. Materials and methods

3.1 In vitro methods

3.1.1 Cell culture

Murine embryonic stem cells (mES cells) were purchased from Merck Millipore AG, Switzerland (Millipore, SCC050). mES cells that expressed the transgenes luciferase and lacZ were purchased at Applied Biological Materials Inc. (Richmond, Canada). The lentiviral vectors for lacZ and luciferase are displayed in the supplements (S1 and S2). The murine induced pluripotent stem cells (miPS cells) as well as the transgenic miPS cells (miPS Luc/Lac) were purchased at Applied Biological Materials Inc. (Richmond, Canada) and were virus free reprogrammed by the company. The plasmid sequences for Oct4, c-myc and Klf4 are displayed in the supplements (S3 and S4). The transgenic cells were additionally equipped with a puromycin resistance gene (S4).

Murine embryonic fibroblasts (mEF) derived from FN-floxed murine embryos were kindly provided by Reinhard Fässler (Max Planck Institute of Biochemistry, Martinsried, Germany).

The isolation of the cell clones miPS Luc/Lac clone 5 (miPS clone 5) and mES Luc/Lac Clone 11 (mES Clone 11) was performed by Cameron Moshfegh (Laboratory of Applied Biomechanics, ETH Zürich, Switzerland) by single cell isolation and selected for expressed transgenes prior to this study. miPS Luc/Lac cells were diluted and seeded in 96-well plates. 96-well plates were then checked for single cells in each well and cultured for several days.

For culturing mES and miPS cells EmbryoMax® ES Cell qualified DMEM (Millipore, SLM220-B) was used as basic medium (BM). The basic growth medium (BGM) contained 15% v/v. of Embryomax® ES qualified fetal bovine serum (Millipore, ES009-C), 1 mM of minimal essential sodium pyruvate solution (Millipore, TMS-005-C), 0.1 mM of non-essential amino acids (Life Technologies, 11140-035) and 2 mM of GlutaMAX™ supplement (Life Technologies, 35050-038). For culturing mES and miPS cells 0.1 mM of β-mercaptoethanol (βME, Life Technologies, 31350-010), 1000 units/ml of LIF (Leukemia inhibitory factor, EMD Millipore, ESG1106), 1 µM of CHIR99021 (Cayman Chemicals, 13122), 1 µM of PD0325901 (Sigma Aldrich, PZ0162) and for transgenic cells 0.5 µg/ml of

puromycin dihydrochloride (Invitrogen, A11138-03) were added to BM (2iL or 2iL + puro respectively).

mEF cells were cultured in 1x DMEM plus GlutaMAX™ (Gibco by Life Technologies, 21885-025) with 9 % v/v of fetal bovine serum (BioWest, S181H).

All cells were stored in a humid environment at 37°C and 5% CO2 in an incubator. mES, miPS and mEF cells were passaged by volume 1:10 – 1:30 every two to three days at around 70-90% confluence. Cells were cultured in T25/T75 cell culture flasks (TPP, Thermo Fischer Scientific, Switzerland 90026/90076), 6-well plates (TPP, Thermo Fischer Scientific, Switzerland 92006) or µ-Slide 8-well dishes (ibidi, Germany 80826). The plates were incubated for 10 minutes with EmbryoMax® 0.1% gelatin solution (Millipore, ES-006-B), except for mEF cells, then the gelatin was removed and the cell suspension was added. Cells were dissociated after incubation for 10 minutes with StemPro® Accutase® Cell dissociation reagent (Invitrogen, A11105-01). The Accutase® reaction was neutralized by adding equal amounts of medium. The cell suspension was centrifuged at 1100 rpm in 15ml Falcon®-tubes for 3 minutes at room temperature. The supernatant was removed and the cell pellets were resuspended in respective media. The centrifugation step was repeated and then cells were diluted with the particular dilution factor in the respective media and added to the new flask/dish. For cell counting, 10 µl of cell suspension was diluted 1:10 in medium and 10 µl of the diluted suspension was pipetted to a Neubauer improved cell counting chamber and cells were counted using a phase contrast microscope at 20x magnification.

For cell freezing, cells were cultured in T25 or 6-well dishes for two days. They were then dissociated with Accutase®, washed twice in the respective medium and then diluted in CryoStor®CS10 (Stemcell Technologies™, 07930) cell freezing medium at 10^6 cells/ml in Cryo.S™ cryotubes (Greiner bio-one, 123 2XX). Cells were then stored in liquid nitrogen.

3.1.2 GoST induction series

For the GoST induction series only miPS clone 5 cells were used. Cells were seeded at 20’000 cells/cm^2 in 8-well ibidi dishes for immunofluorescence, 3 wells of one 6-well dish in triplicates for real-time RT-PCR and in three T25 flasks for Hematoxylin & Eosin (H&E) staining of cell-pellets. The series contained two groups. The first group consisted of miPS clone 5 cells that underwent GoST induction (GoST cells). The second were miPS clone 5

(WT) cells that did not undergo the GoST induction as a negative control. In the first group, miPS clone 5 cells were cultivated in 6-well dishes over 10 passages in 2iL + puro with additional 5 µM of Ex527 (Cayman Chemical Company, 10009798) and 100 µM of RG108 (Cayman Chemical Company, 13302), both diluted in cell culture grade dimethyl sulfoxide (DMSO, Applichem, A3672,0100). The resulting cells were described as conditioned miPS clone 5 (cmiPS clone 5) cells. In the second group, WT cells were cultured in a T25 flask with 2iL + puro plus 0.1 µl/ml DMSO for 10 passages. These cells were described as WT cells. After 10 passages the complete medium of cmiPS cells was changed from 2iL + puro plus Ex527 and RG108 with BM with added EX527, RG108 (in unchanged concentrations) and 10 µM tert-butylhydroquinone (tBHQ, Sigma-Aldrich, 112941-5G). cmiPS cells were cultured under these conditions for seven days without splitting, but exchanging the media every 10-16h. The cmiPS-derived cells were described as GoST cells after the seven days. Culturing of WT cells was synchronized by starting at a later time point. After the conditioning step, the cmiPS cells were seeded into 8-well ibidi dishes for immunofluorescence and 6 well plates for mRNA isolation. WT cells were seeded into 8-well ibidi dishes and 6 well plates after 10 passages. Then the media of both WT and GoST cells were exchanged completely with BM plus 0.1 mM βME for additional 20 days. In those 20 days the volume of medium was raised to 600 µl/well in 8-well ibidi dishes and 6 ml/well in each well of the 6-well dishes. The covers of the 8-well dishes were removed and the dishes were put in a 137 mm round cell culture dish (TPP, 93150 2/3). 400 µl of medium of a total volume of 600 µl were changed twice per day in the 8-well dishes after 12-16h incubation time in the morning and 8-12h in the evening. In the 6-well dishes 4ml of medium of a total volume of 6 ml were changed twice per day after 12-16h in the morning and 8-12h in the evening. In the T25 flasks the volume of the medium was raised to 8 ml total and 6 ml of medium was exchanged twice per day after 12-16h incubation time in the morning and 10-12h in the evening. The cells were harvested for RNA isolation and fixed for immunofluorescence at specific time points. For GoST cells the starting point was the start of the GoST induction (Conditioned D0) and after the 7 days of GoST induction every 4 days, starting with GoST D7 following GoST D7+4, GoST D7+8, GoST D7+12, GoST D7+16 and GoST D7+20. Harvesting of WT cells started at the day of medium exchange from 2iL + puro plus DMSO starting with WT D0 following WT D0+4, WT D0+8, WT D0+12, WT D0+16 and WT D0+20. From harvesting points GoST D7 and WT D0+4 on cells were harvested with a cell scraper due to their adhesiveness. The scraped

cells were diluted in 5 ml 1x PBS pH 7.4 (Gibco® by Life Technologies, 10010-015) and centrifuged at 1100 RPM. Then the supernatant was discarded and the pellet was resuspended in 1 ml of PBS and centrifuged again. The resulting cell pellets were used for RNA isolation. For fixation, the complete medium of each well was removed and the cells were washed twice with 1x PBS, then fixed with 2.5% Formaldehyde in PBS for 10 minutes. The Formaldehyde was then exchanged for 1x PBS and the dishes were stored at 4°C.

3.1.3 Immunofluorescence

Fixed cells in 8-well ibidi dishes were washed once with PBS and permeabilized with 0.3% Triton X-100 (Sigma, T8787-50ML) in PBS for 10 minutes at room temperature (only for intracellular staining) and washed again once with PBS. Then they were blocked for 1h with 3% bovine serum albumin (BSA, Sigma, A4503-50G) in PBS at room temperature. Samples were then incubated with the primary antibody, diluted in 3 % BSA, overnight at 4°C. The primary antibodies were anti-Nanog (0.5 mg/ml, 1:200, mouse, BD Biosciences, 560259), anti-Sox2 (1 mg/ml, 1:200, mouse, Millipore, MAB4343), anti-TRA98 (1 mg/ml, 1:200, rat, Abcam, ab82527), anti Oct4 (1 mg/ml, 1:200, rabbit, Abcam, ab19857), anti-SSEA1 (1 mg/ml, 1:200, mouse, Millipore, MAB4301), anti-Gfra1 (1 mg/ml, 1:200, rabbit, Santa Cruz, sc10716), anti-firefly luciferase (1 mg/ml, 1:200, mouse, Abcam, ab16466). Then cells were washed once with 1x PBS for 5 minutes and, if necessary, washed twice for 5 minutes with 0.1% Triton X-100 in PBS. Then they were washed again twice with PBS for 5 minutes. Afterwards the cells were incubated with the secondary antibody, diluted in 3 % BSA for 1h at room temperature. The secondary antibodies were anti-mouse IgG conjugated to Alexa Fluor 488 (2 mg/ml, 1:400, donkey, Life Technologies, A-21202), anti-rat IgG conjugated to Alexa Fluor 546 (1 mg/ml, 1:200, goat, Molecular Probes, A11081), anti-rabbit IgG conjugated to Alexa Fluor 555 (1 mg/ml, 1:200, goat, Invitrogen, A-21429), anti-mouse IgG conjugated to Alexa Fluor 633 (1 mg/ml, 1:200, goat, Invitrogen, A-21052), anti-rabbit IgG conjugated to Alexa Fluor 647 (1 mg/ml, 1:200, goat, Life Technologies, A-21245) and anti-rat IgG conjugated to DyLight™ 649 (1 mg/ml,1:200, donkey, Jackson Immuno Research, 712-495-153). Then the cells were washed once with PBS for 5 minutes and for intranuclear staining twice with 0.1% Triton X-100 for 5 minutes. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, 28718-90-3) at a dilution of 2.5µg/ml for 10

minutes. Cells were mounted using ProLong® Gold Antifade Mountant (Life Technologies, P10144) and covered with an approximately 9x9 mm microscope cover glass cut from a 18x18 mm microscope cover glass No. 0 (Marienfeld, 0100032) and dried at room temperature overnight inside a drawer. For TRA98 staining all incubation times were doubled.

Imaging was performed with a Leica TCS SP5 confocal microscope (Leica, Microsystems, Mannheim, Germany). DAPI was excited with a 405nm laser with gain from 500-700V and emission was detected between 425-480nm. Alexa Fluor 488 was excited with a 488nm laser with gain between 736-850V and emission was detected between 505-550nm. Alexa Fluor 546 was excited with a 561nm laser with gain of 700V and emission was detected between 571-600nm. Alexa Fluor 555 was excited with a 561nm laser with gain of 901V and emission was detected between 571-605nm. Alexa Fluor 633 was excited with a 633nm laser with gain of 908V and emission was detected between 643-670nm. Alexa Fluor 647 was excited with a 633nm laser with gain of 725V and emission was detected between 653-695nm. For excitement of Dylight™649 a 633nm Laser was used with gain of 900V and emission was captured between 656 and 690nm. Images of luciferase were taken with a 20x air objective. All other images were taken with a 63x PL APO CS 1.4 oil objective. For analysis and optimization of images taken ImageJ, Adobe® Illustrator CS6 and the Leica LASAF software were used. Size and brightness of the pictures were altered solely and identically for each group compared.

3.1.4 Light microscopy

For light microscopy mES, miPS, miPS clone 5, mEF and the GoST series cells were cultured either in 8 well dishes or T25 flasks. The medium of the cells was completely removed and cells were washed twice with 1x PBS, then fixed with 2.5% Formaldehyde for 10 minutes. Afterwards the Formaldehyde was exchanged with 1x PBS and cells were kept at 4°C in a refrigerator until imaging. For light microscopy an Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany), with either 10x Plan-Neofluar 0.3 Ph1 or 20x LD Plan-Neofluar 0.4 Ph2 objectives was used. For analysis and optimization ImageJ, Adobe® Illustrator CS6 and the AxioVision Rel. 4.8 software were used. Size and brightness of the pictures were altered solely and identically for each group compared.

3.1.5 Luciferase assay

For the Luciferase assay miPS Luc/Lac and miPS clone 5 cells were thawed and cultivated in 4 wells of an 8-well dish each at a density of 34'000 cells/cm^2 with 2iL + puro for two days. Cells were then treated with the Luciferase Assay System with Reporter Lysis Buffer (Promega, E4030) according to the manufacturer's protocol. Analysis was performed using a Tecan Infinite M200 (Tecan, Switzerland). Readouts were analyzed using Microsoft Excel.

3.1.6 X-Gal staining

For X-Gal staining miPS Luc/Lac and miPS clone 5 cells were cultivated in two wells of an 8-well dish each, at approximate 34'000 cells/cm^2 cell density in 2iL + puro for 2 days. Then media were removed completely and cells were washed three times with 1x PBS. The X-Gal staining was conducted according to the manufacturer's manual. Images were taken with the Axiovert 200M inverted microscope with 10x air and 20 x air objectives with 59ms exposure time for 10x images and 41ms (miPS clone 5) and 30ms (miPS Luc/Lac) exposure time for 20x images. Images were analyzed and optimized using ImageJ and Adobe® Illustrator CS6. Only size and brightness of the images were altered solely and identically for each group compared.

3.1.7 Real-time PCR analysis

Total RNA was isolated from ~ 1x10^6 cells per sample using the RNeasy Mini Kit (Qiagen, 74104/74106) with RNAse free DNAse treatment (Qiagen, 79254), following the manufacturers manual. RNA concentration was measured with a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). Isolated RNA was stored in 500 µL RNase-free tubes provided by the kit at -20°C.

After measurement of RNA concentration, RNA was diluted to a total amount of 2 µg or 1 µg in 15 µl in Nuclease-free water (Life Technologies, 10977-035) into 500 µl thin walled

Eppendorf PCR tubes (Eppendorf, 0030124537). For reverse transcription (RT) 2 µg total RNA was reverse transcribed into cDNA in a reaction volume of 20 µl using the iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, 1708842). Each RT reaction mix contained 4 µl 5x iScript advanced reaction mix and 1 µl iScript advanced reverse transcriptase. The 20 µl reactions were incubated in an Eppendorf Mastercycler (Vaudaux, Switzerland) at 42°C for 30 minutes, at 85°C for five minutes and held at 4°C. The resulting cDNA was then diluted 1:10 (2 µg samples) or 1:5 (1 µg samples) in nuclease-free water and stored at -20°C.

DNA was isolated from mES, miPS Luc/Lac, miPS clone 5 and mouse tissues (ear-punches, liver, lung, spleen, tumor) with the DNeasy blood and tissue kit (Qiagen, 69504/69506) following the manufacturers protocol (page 28-30). For liver, lung and spleen samples an additional amount of 20 µl of Proteinase K was added and the incubation time of Proteinase K digestion was extended to 24h. For the digestion step, samples were put in an Eppendorf Thermomixer for 24h at 500 rpm. DNA concentration was measured using the NanoDrop UV Spectrophotometer. Samples were stored in 1.5 ml Eppendorf tubes at -20°C.

DNA and cDNA for real-time RT-PCR analysis were diluted 1:10 in nuclease-free water. The Master mix for each reaction contained 10 µL SsoAdvanced™ SYBR® Green Supermix 2x (BioRad, 172-5262), 5 µL sample with a total amount of 5 ng cDNA, 3.8 µL nuclease-free water, 0.6 µL forward primer and 0.6 µL reverse primer to a total volume of 20 µL. PCR reactions were performed in Hard-Shell® Low-Profile Thin walled 96-Well Skirted PCR plates (Bio-Rad, HSP-9602). Primers were designed using PrimerSelect from the Lasergene software suite (DNASTAR, Madison, WI). All primers were synthesized at Microsynth (Mycrosynth, Balgach, Switzerland). Gene abbreviations, NCBI reference, amplicon size and primer sequences are indicated in table 1.

Table 1

Gene	mRNA ID (NCBI Reference Sequence)	Amplicon Size (bp)	Forward Primer Sequence 5' → 3'	Reverse Primer Sequence 5' → 3'	Species
Reference Genes					
Tbp	NM_007393	265	GCTGTATTCCCCTCCATCGTGG	CACGGTTGGCCTTAGGGTTCAG	Mus musculus
Gapdh	NM_008084	90	ACCCCCAATGTGTCCGTCGTG	AGATGCCTGCTTCACCACCTTCTTG	Mus musculus
Core Pluripotency Markers					

Nanog	AB093574	101	ACCTCAGCCTCCAGCAGATGCAA	CCGCTTGCACTTCACCCTTTGG	Mus musculus
Oct4	NM_013633	135	TGCTGAAGCAGAAGAGGATCACCTTG	TGTTCTTAAGGCTGAGCTGCAAGGC	Mus musculus
Sox2	NM_011443.3	139	AGGGTTCTTGCTGGGTTTTGATTCTG	AACGGTCTTGCCAGTACTTGCTCTCAT	Mus musculus
Tbx3	NM_011535	127	CGCCCTGTCCCTTTCAGTTTTGTC	AGTCCCGCGTTTCAAAGCAACAG	Mus musculus
Klf4	NM_010637	111	GCCATTATTGTGTCGGAGGAAGAGGA	GCTCCCCCGTTTGGTACCTTTAGAAC	Mus musculus
Esrrb	NM_011934	262	TGCCCGGGACCCAAGAGACATA	AGTGAGTTCCGGCTGGCTGAGGT	Mus musculus
Nr5a2	NM_030676	152	CGCATGGGAAGGAAGGGACAAT	CCGCTGATCGAACTGAAGGGAAC	Mus musculus
Naïve Pluripotency Markers					
Prdm14	NM_001081209	113	CGCCACCACCGAGGAGGAGT	CCGGGTTCAACAAGGGAGCAGT	Mus musculus
Dppa3	NM_139218	181	TGCAGCCGTACCTGTGGAGAACA	GTCCCGTTCAAACTCATTTCCTTCG	Mus musculus
Stra8	NM_009292	130	CAGCGCTATGTTTGCCACCTGC	TGGGGGCTCTGGTTCCTGGTT	Mus musculus
Dazl	NM_010021	262	ACCTCCGGCTTATACAACTGTTAACTACCA	AAGCACTGCCCGACTTCTTCTGAA	Mus musculus
Rex1	NM_009556	144	TGGCTGCGAGAAGAGCTTTATTCAGTC	CGTGTATCCCCAGTGCCTCTGTCAT	Mus musculus
Dax1	NM_007430	115	GCGAGTGGTGGCAGCTGTCCTAC	GGCTGCTCTTCACCGCACACATAG	Mus musculus
Piwil2	NM_021308	152	TCCGCAAGGACAGAGAAGAACCC	TGCTCGTCCCAGTGGTAACAGAGAG	Mus musculus
Fbxo15	NM_015798	125	GTGGGGCTGTGGCAGGAGAATG	GTAGTGTCGGGAGGCAATGTATAGGGAA	Mus musculus
Germ cell Markers					
Nanos2	NM_194064	76	ACCGGCGACCAGGCTCATACAC	GCCCACTGCGTCGGTAGAGAGACT	Mus musculus
Tdrd1	NM_001002238	244	GTGGCCTGGCAGAAAACCTCACTT	CTGGCGTTTGCTGTCTCTTTCTTCC	Mus musculus
Ddx4	NM_010029	100	AACGCCAAACCCTTTTATTCAGT	TGCCCAACAGCGACAAACAAGTAA	Mus

			GCTAC	CT	musculus
Zbtb16	NM_001033324	86	CCGCCCATTTTTACCCCCTACAA	ACCCCAGCCCATATCCTCTCAACA	Mus musculus
Tex101	NM_019981	225	CTAATCGCCTCACGTTGGACTCTGG	CACCGCCTCTCCTCCTTGAGAAAC	Mus musculus
Plk1s1	NM_001033298	132	AGATTCGCTCTTCACGGAATATGTTTTACA	CATTGCCCTCGTGTCTTCAATAAGTGAC	Mus musculus
Spermatogonial stem cell Markers					
Gfra1	NM_001285457	188	TGCCACGACTACCACTGCCTTCC	CAGCGAGACCATCCTTTCCGTAATC	Mus musculus
Csf1r	NM_001037859	136	ATGCCCGCCTGCCTGTAAAGTG	ATGCCGGGGTAGGGGTTCAGAC	Mus musculus
Cd53	NM_007651	279	TGCATGGGCTCAATCAAGGAAAATAA	TGATGGGCAGGAAGATGGTGGAC	Mus musculus
Ifi203	NM_001302649	248	TTGGGTGCAATGGGTTTCTGGAG	AACCACCACTGCCATTTTTCCTGTATC	Mus musculus
Tyrobp	NM_011662	77	GAGCCCTCCTGGTGCCTTCTGTT	TCACTCTGGGCCTGTACGGGACTTA	Mus musculus
Col1a2	NM_007743	115	AGGGCAACAGCAGGTTCACCTACTCT	AACGGCAGGCGAGATGGCTTATT	Mus musculus
Transgenes					
Luc		202	ATTCCGGATACTGCGATTTTAAGTGTTGTT	CCAGGGGTTGCCATTCTCATCATACT	Photinus Pyralis
LacZ		83	AGTACCCCAGGCTGCAAGGAGGAT	TGCTTTTGGCGAAGAAGGAGAATAGG	E. coli
Sex Markers					
Xist	NC_000086.7	136	ACCCCGCAAATGCTACCACAAATC	TTACGCCAAGAATTAGGACACCGAGG	Mus musculus
Sry	NC_000087.7	118	AAAGGGCCTTTTTTCGGCTTCTGTAA	GTCCCGTGGTGAGAGGCACAAGTT	Mus musculus
Zfy	NC_000087.7	197	AGCCGCCATCTTGCCTCGTG	AGCCGCAATCACCGTGCTTAGTAAAT	Mus musculus

Real-Time RT-PCR reactions were performed on DNA and cDNA using CFX Connect™ Real-time PCR Detection System (Bio-Rad). PCR reactions were incubated at 95°C for 3 minutes, followed by 39 cycles of 95°C for 10 seconds and 62°C for 30 seconds and were run as triplicates of three independent experiments. Cycling protocols, plate setups and master mix were designed using the BioRad CFX Manager 2.1 software (Bio-Rad). Readouts were

analyzed and optimized using Microsoft Excel and BioRad CFX Manager 2.1. mRNA expression levels relative to Gapdh and Tbp were calculated based on the ΔΔCt method. Statistical analysis was conducted using the ANOVA shiny app for a One-Way ANOVA (http://shiny.stat.tamu.edu:3838/hassaad/SumAOV1/). For ANOVA analysis the standard error of the mean (S.E.M.) and a Tukey/Kramer test were used.

3.1.8. Western blot analysis

For Western blot analysis mES, miPS and mEF cells were cultured in triplicates under standard culture conditions in three wells of a 6-well dish for each cell line. After two days the medium was removed and cells were washed twice with PBS. Then freshly mixed RIPA buffer (Sigma-Aldrich, R0278) combined with both protease and phosphatase inhibitors (Sigma-Aldrich) were added. The dishes were incubated for 5 minutes on ice. Cells were then scraped from the surface with a cell scraper and cell lysates were pipetted into an Eppendorf tube on ice. Samples were then centrifuged for 10 min at 10'600g. The supernatant was pipetted into another Eppendorf tube and 20 µL of the supernatant was stored at -20°C for protein concentration measurement using the Pierce BCA protein assay (Thermo Scientific, 23225). Afterwards the exact volume of remaining supernatant was measured and incubated with 5x Laemmli buffer for 5 minutes at 95°C with an Eppendorf Thermomixer. Samples cooled down to room temperature on a bench and were then stored at -20°C until blotting.
Samples were loaded with 10 mg/well, fractionated by a 6% stacking gel and 10% running gel SDS-PAGE and transferred to a methanol activated polyvinylidene difluoride (PVDF) membrane. The membrane was incubated in 10x Roti®-Block (Roth, A151.1) TBS-T buffer overnight at 4°C in a 50 ml tube on a multi-axle-rotary-mixer CAT-RM5 (Ingenieurbüro M. Zipper GmbH, Staufen, Germany) with the primary antibody. The primary antibodies were anti-Nanog (1:1000, mouse, BD Biosciences, 560259), anti-Oct4 (1:1000, rabbit, Abcam, ab19857), anti-Sox2 (1:1000, mouse, Millipore, MAB4343) and α-tubulin (1:1000, mouse, Abcam, ab7750). The membrane was washed three times with tap water, once with TBS-T for 10 minutes, blocked with Roti® buffer and incubated with the secondary antibody for 1h at room temperature. The secondary antibodies were anti-rabbit horse radish peroxidase (Jackson ImmunoResearch Laboratories, 1:2000, donkey, 711-035-152), anti-mouse alkaline peroxidase (Promega, S3721, 1:2000) and anti-mouse HRP (Jackson ImmunoResearch

Laboratories, 1:2000, donkey, 715-035-150). Blots were further developed following the manufacturer's protocol using ECL plus substrate (GE Healthcare, Piscataway, NJ, USA) or CDPstar substrate (Sigma-Aldrich, C0712). Blots were developed in a darkroom on Fujifilm Super RX medical X-Ray film 100NIF 18x24 (Fujifilm, 47410 19236). Films were scanned and analysis was performed with ImageJ and Microsoft Excel.

Western blot experiments were conducted under supervision and aid from Lina Aires (Laboratory of Applied Mechanobiology, ETH Zurich).

3.1.9 Chromosome painting

For Chromosome painting mES, miPS and miPS clone 5 cells were cultured under standard culture conditions in one well of a 6-well dish each. Cells were scraped from the surface and transported in BGM to Claude Schelling (Clinic of Reproductive Medicine, Animal Genetics Group, Vetsuisse-Faculty University of Zurich) and Dr. eng. Aldona Pieńskova-Schelling (Institute of Genetics, Vetsuisse-Faculty University of Bern, Bern, Switzerland).

Alive mouse cells were arrested in the metaphase stadium by adding the Colcemide (0.5 ml for each 10 ml of culture medium) for 2h. After delicate centrifugation (800 rpm, 8 min, +24°C) and removing the supernatant hypotonic KCl solution (0.075M, +37°C) was added by slow dropping till 6 ml and then cells were incubated 20- 25 min at +37°C. After incubation, centrifugation and removing the supernatant the fixation of cells was performed by slow dropping a frozen (-30°C- -80°C) Carnoy fixative (absolute methanol and glacial acetic acid in proportion 3:1) until 8ml, centrifugation at +4°C, 800 rpm, 8 min, then after removing the supernatant again the fixative was added until 4ml. The chromosome suspensions were concentrated to 100 µl and 10 µl of it were dropped onto cold, wet microscope slides. Slides were cleaned in the detergent before usage, rinsed in distilled water and incubated in 2N HCl for 2-3 days. Then rinsed again in distilled water and stored in the absolute ethanol at -20°C. All materials were dropped at one place that the surface area was not bigger than Ø 15mm. The microscopic preparations were prepared 24h before hybridization and incubated in this time in dry environment at +37°C. The hybridization was carried out for 18-24h.

In a first step RNA was digested by reaction with RNAse A (100-120 µl RNA-se from working solution: 25 µl RNA-se A 10mg/ml + 2.475 ml 2x saline-sodium citrate buffer (SSC) which was carried out in humid environment for 1h at +37°C. After incubation, slides were

rinsed in a 2x SSC at room temperature, 3x for 2.5 min and then dehydrated by incubation in the ethanol row 70%-80%- 90% for 2.5 min in each concentration at room temperature. Next steps were carried out exactly according to the manufacturer's instructions from Chrombios (Nussdorf, Germany) multi-color FISH (http://wp.chrombios.de/about-fish/multi-color-fish/). For image acquisition, the microscope Axio Imager Z1and the MetaSystems software ISIS/IKAROS was used.

3.2. In vivo methods

3.2.1. Teratoma formation assay

One of the main goals of this study was to evaluate the tumor growth potential of miPS-derived GoST cells. The gold standard to assess the potential tumor growth is the teratoma assay. PS cells proliferate *in vivo* into multi lineage differentiated tumors, called teratoma, that consist of all three germ layers when transplanted into an immunocompatible host. For our teratoma assay we chose the NSG mouse model since it represents one of the most immune compromised mouse strains and therefore was ideal for transplantation experiments. WT and GoST cells were derived from miPS clone 5 cells.

The teratoma formation assay was conducted under the animal license number 166/2014. For the teratoma formation assay 16 weeks old female white NSG mice (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ, Charles Rivers, Saint-Germain-Nuelles, France) in six groups with five mice per group were used. The animals were held at 21°C (+/-1°C) and 50% rel. humidity (+/- 10%) in individual ventilated cages (IVC's) Type II long (Fa. Bioscape, Emmendingen, Germany) with LIGNOCEL select bedding (Fa. JRS, Rosenberg, Germany) in IVC rack systems. The mice were fed with autoclaved pellets for mice and rats (Fa. Kliba Nafag, Kaiseraugst, Switzerland) and watered with acidified and autoclaved tap water to a pH between 2.75 and 3.5. For acidification 25% sulfuric acid was used. Identification of each mouse was facilitated by earmarks from one to five for each cage. Ear punches were collected for DNA isolation and subsequent Real-Time PCR analysis. Injected cells consisted of GoST cells D7 or WT cells D0 respectively in two T75 flasks each. The preparation of the cells was provided by Dr. Cameron Moshfegh (Laboratory of Applied Mechanobiology, ETH Zurich).

Cells were dissociated, washed and resuspended in a 1:1 Corning® Matrigel® hESC-qualified matrix (Corning, 354277) PBS mixture containing either 10^2, 10^4 or $3*10^6$ cells on ice for each group of 5 mice. Mice were previously shaved and injected under 2.5 Vol% Isoflurane volatile anesthesia (Attane™, Primal Healthcare Limited, India) on a heating pad at 37°C with 100µL of the Matrigel/PBS/Cell mixture subcutaneously into the upper left abdominal wall approximately 5mm laterally from T12-L2 vertebrae, near the caudal end of the ribcage. Two mice had to be injected into the right upper left abdominal wall due to previous injection errors. Mice were put back into their cages after waking up and were observed three times a week on behavior, habitus and eventual tumor growth for 13 weeks. Additionally, once per week each mouse was taken out of the cage and examined individually, including palpation of the injection site and measurement of the tumor growth using a sliding caliper. If the tumor diameter gained over 15mm in diameter and/or the behavior of a mouse was heavily impaired by the tumor growth, the mouse had to be sacrificed. End points for each mouse were determined by a score sheet (S5 and S6). Mice that reached the end point were discharged separately in individual cages from the animal facility followed by IVIS imaging.

3.2.2 IVIS Imaging

Discharged mice were weighted and injected with a dosage of 150 µg/kg bodyweight of optimized D-Luciferin (Perkin Elmer, 122796) intraperitoneally. After 5 - 10 minutes the mice were put into Isoflurane 2.5 Vol% volatile anesthesia and put into the IVIS. Prior to the IVIS imaging process, the region around the tumor was shaved. The mice were kept under anesthesia during the whole imaging process. Pictures were taken with a binning factor of 2 and an exposure time of 1 sec with a delay of 1 min between each consecutive image. Four pictures were taken for each mouse. Immediately after the imaging process each mouse was sacrificed by cervical dislocation while still under anesthesia. Immediately afterwards blood was drawn from the heart with an EDTA wetted syringe and stored in an Eppendorf tube. The corpses were put on ice and after every mouse was imaged a dissection was performed on each mouse. At first, the abdominal cavity was opened and liver, spleen, kidney and ovaries were removed. For liver samples tissue from the *Lobus lateralis dexter* was taken. For Spleen samples a part of the cranial pole was taken. Tissue from the kidney was obtained from half of the left kidney. Afterwards the chest was opened and lung and heart were extracted from the

body and parts of the organs were stored at -80°C. For lung samples a part of the *Lobus cranialis sinistri* was collected. Samples from the heart were obtained from the caudal apex of the heart. Then, bone marrow from the right femur was extracted. In the end the skin with the tumor was removed and parts of the chest skin and tumor were stored. All samples were stored at -80°C. The remains of the mice were fixated in neutral-buffered 10% formalin. Tumor parts for histological analysis were incubated in formalin for 48 hours and then the formalin was exchanged for 1x PBS. All fixated samples were sent to a pathologist for histological analysis. For some mice, parts of the organs were frozen in O.C.T.™ compound (Sakura Finetec Europe B.V., 4583) and stored at -80°C for histological analysis.

3.2.3 Tumor pathology and histological analysis

Tumor pathology and histological analysis was conducted by Giovanni Pellegrini at the Laboratory for Animal Model Pathology (LAMP, Vetsuisse Faculty University of Zurich). A complete necropsy, including a thorough external and internal gross post mortem examination was performed on each mouse. The injection site area, including the subcutaneous mass and the adjacent skin, was sampled and fixed in formalin for histological examination. After 48 h fixation in formalin, the masses from all animals were trimmed through their longitudinal axis, dehydrated in graded alcohol and routinely paraffin wax embedded. Sections (3-5 µm thick) were prepared, mounted on glass slides, deparaffinized in Xylene, rehydrated through graded alcohols and stained with Haematoxylin and Eosin (HE) for the histological examination. In a few mice euthanized in the early phases of the experiment, the masses were entirely frozen using liquid nitrogen and stored at -80°C. These were evaluated by an European College of Veterinary Pathologists (ECVP)-certified veterinary pathologist in a blind fashion.

For histological analysis, the slides were scanned with a Hamamatsu NanoZoomer-XR Digital slide scanner (C12000, Hamamatsu Photonics, Hamamatsu, Japan) and analyzed using the NDP.view2 viewing software (U12388-01, Hamamatsu Photonics, www.hamamatsu.com/jp/en/U12388-01.html).

4. Results

4.1 miPS clone 5 expresses core pluripotency markers and the introduced transgenes

Since it has been shown that mES and miPS cells differ in expression patterns[32] our aim was to evaluate the expression of pluripotency factors in ES and iPS cells. The GoST induction has been established for mES cells[1], but not for miPS cells. Significant differences in pluripotency of mES and miPS cells potentially influence the outcome of the GoST induction. The germ cell-specific marker TRA98 was compared between mES and miPS clone 5 since the GoST induction induces gonocyte-like cells in mES cells. The isolated miPS clone 5 was selected from miPS Luc/Lac cells for expression of the introduced transgenes and did show the highest level of all isolated miPS Luc/Lac clones. Therefore miPS clone 5 represents the ideal cell clone in terms of reporter gene expression for this study (Fig. 1 and Fig. 2). We used mEF cells as a negative control for pluripotency since mEF cells represent differentiated cells and thus we did not expect expression of pluripotency factors.

The results of real-time RT-PCR confirmed that mES and miPS clone 5 cells did express the majority of core and naïve pluripotency markers without significant differences (Fig. 1 and 2). The expression of the core pluripotency markers *Nr5a2* and *Tbx3* showed significant differences between mES and miPS clone 5 cells (Fig. 1). Notably the essential pluripotency markers *Nanog*, *Oct4*, *Sox2* and *Klf4* were evenly expressed with no significant differences in mES and miPS clone 5 cells on mRNA expression levels. Expression of naïve pluripotency markers showed significant differences for *Dax1*, *Dazl*, *Stra8* (Fig. 2) between mES and miPS clone 5 cells. mES cells showed higher expression for *Dazl* and *Stra8* compared to miPS clone 5 cells, while miPS clone 5 cells showed higher expression of *Dax1* compared to mES cells. mEF cells did not express most of the core and naïve pluripotency markers (Fig. 1, 2). Analysis of the western blots showed significant differences in Oct4 and Sox2 expression (Fig. 3A) between mES and miPS clone 5 cells. mEF cells showed significant lower expression for Nanog, Oct4 and Sox2 (Fig. 3A). The blots showed a fading signal towards one side of the gel for Nanog, α-tubulin and Oct4 (Fig. 3B).

Immunofluorescence confirmed the previous findings. mES and miPS clone 5 cells did express the core pluripotency markers Nanog, Oct4 and Sox2 and the naïve pluripotency marker SSEA1 (Fig. 4). miPS clone 5 cells showed a brighter signal for Nanog and SSEA1 compared to mES cells. The Nanog signal in miPS cells was more heterogeneous compared to mES cells though. The TRA98 staining showed heterogeneity in mES and miPS clone 5 cells (Fig. 4). mEF cells did not express any of the investigated markers in the immunofluorescence.

The last goal of characterizing the miPS clone 5 cells was the confirmation of the expression of the introduced transgenes. We used immunofluorescence, X-Gal staining and a luciferase assay. The results showed nicely that luciferase is highly expressed in the transgenic miPS clone 5 cells compared to non-transgenic miPS cells (Fig. 5 and 6). Intensive staining for β-Galactosidase in miPS clone 5 cells confirmed these results (Fig. 5).

The characterization confirmed that miPS clone 5 cells show pluripotent characteristics and express the introduced transgenes in equal amounts compared to mES cells.

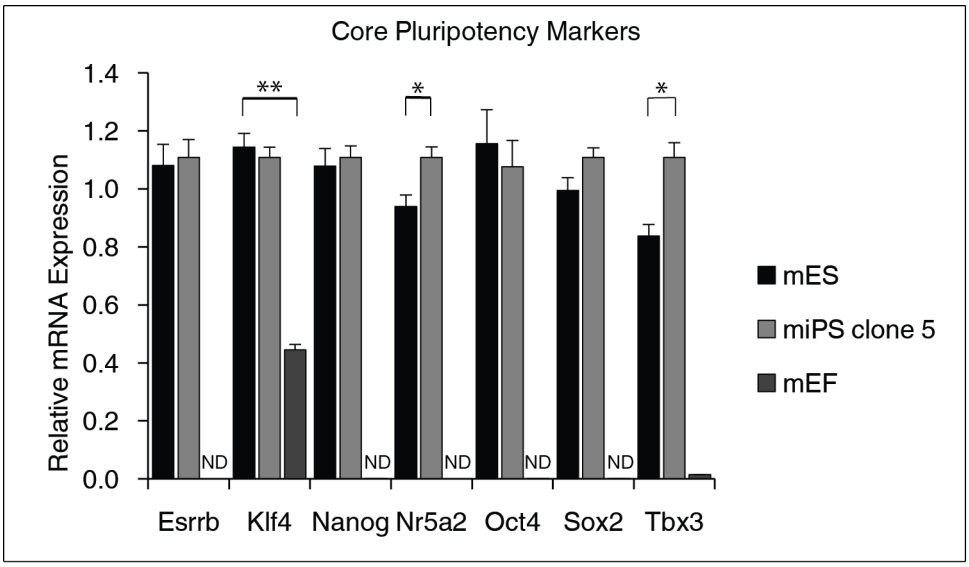


Fig. 1

Real-Time RT-PCR for detection of core pluripotency markers, normalized against TATA Box Binding Protein (Tbp) as reference gene. Error bars correspond to the standard error of the mean (S.E.M.). Data were generated from duplicates of three independent experiments. One star represents $p<0.05$, two stars represent $p<0.01$, three stars represent $p< 0.001$.

Except for Nr5a2 and Tbx3, no significant differences in expression between mES and miPS clone 5 cells were detected. In mEF cells only Klf4 and Tbx3 were expressed at detectable levels but significantly lower compared to mES and miPS clone 5 cells.

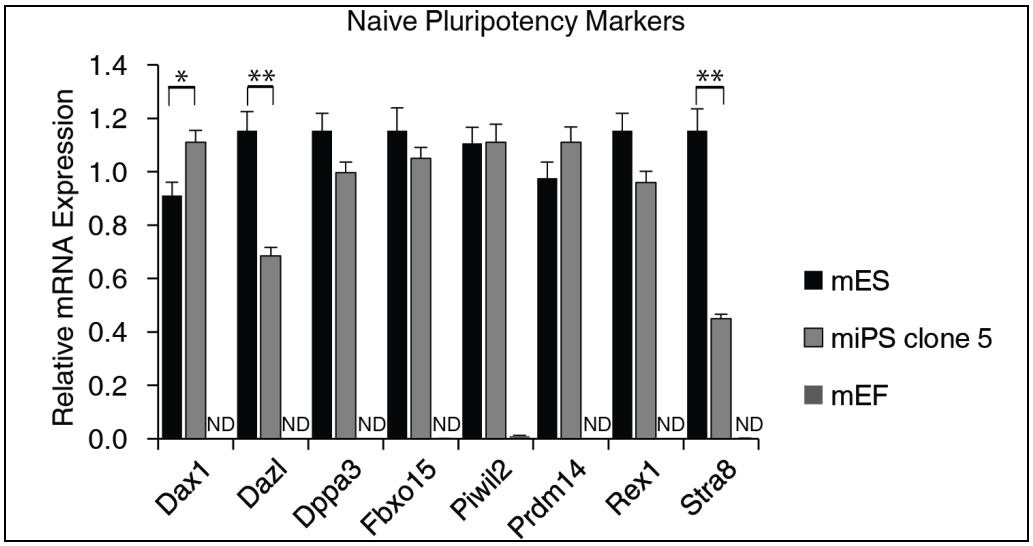


Fig 2

Real-Time RT-PCR for detection of naive pluripotency markers, normalized against Tbp as reference gene. Error bars correspond to the S.E.M. Data were generated from duplicates of three independent experiments. One star represents $p<0.05$, two stars represent $p<0.01$, three stars represent $p< 0.001$.

The majority of naïve pluripotency markers were expressed evenly in mES and miPS clone 5 cells. Expression of Dax1, Dazl and Stra8 differed between mES and miPS clone 5 cells. Expression of Dazl was almost doubled in mES cells compared to miPS clone 5 cells. Dax1 expression was significantly higher in miPS clone 5 cells compared to mES cells. For Rex1 the p-value was close to a one star significance with a p-value of 0.054. Stra 8 expression was found to be over two times higher in mES cells compared to miPS cells. mEF cells did only express Piwil2 at detectable levels but lacked detectable expression of other naïve pluripotency markers.

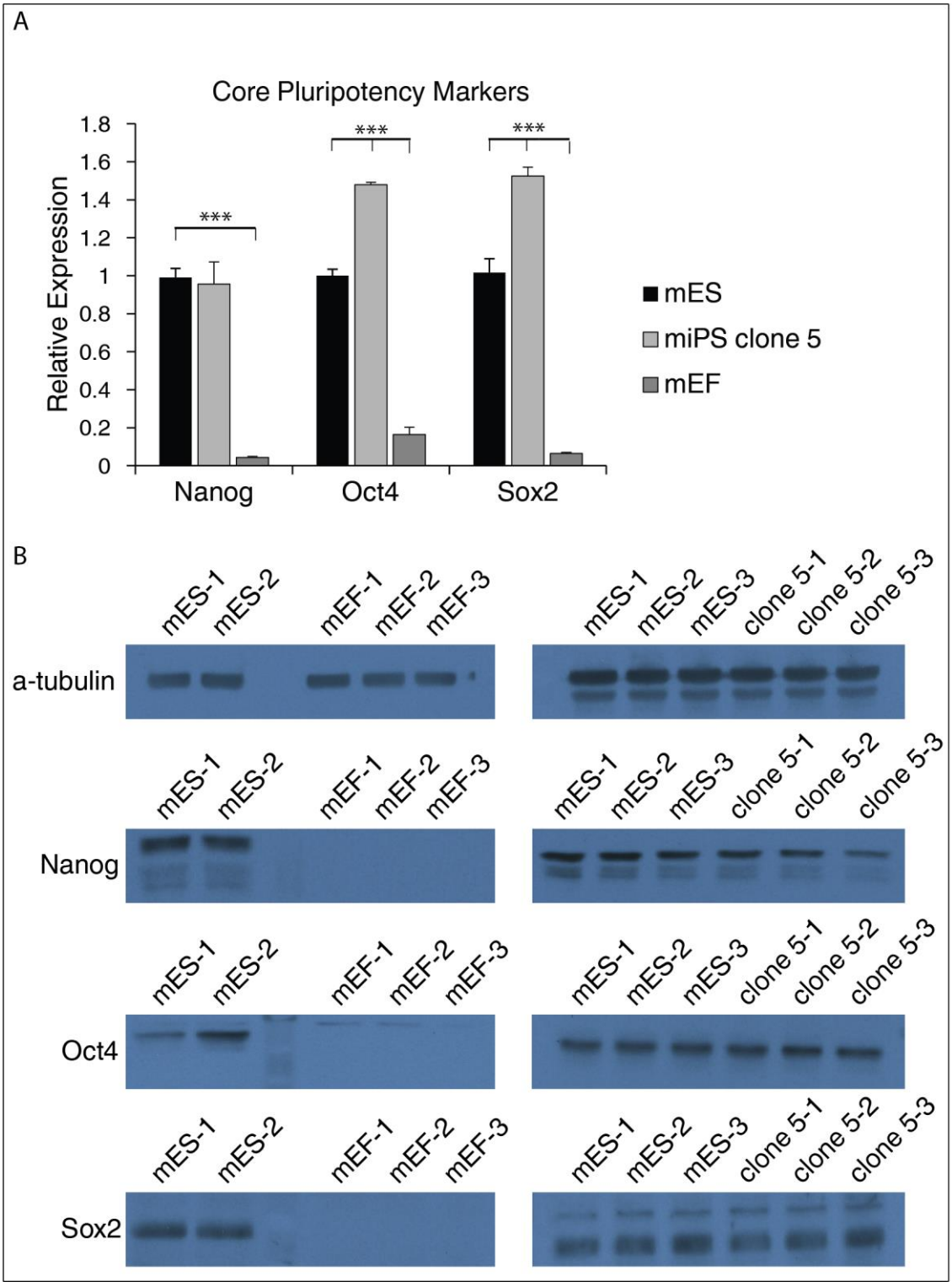


Fig. 3

(**A**) Western blot analysis of core pluripotency markers, normalized against α-tubilin as reference gene. Error bars correspond to the S.E.M.. Samples were analyzed in triplicates. One star represents $p<0.05$, two stars represent $p<0.01$, three stars represent $p<0.001$.

In contrast to the similar expression of Nanog in mES and miPS clone 5 cells, Oct4 and Sox2 show significant differences between the three groups. Expression in mEF is 10-20-fold lower in comparison to mES or miPS clone 5 cells. Oct4 and Sox2 expression is significantly higher in miPS clone 5 cells compared to mES cells, with an approximate 1.5 fold difference.

(**B**) Scan of Western blots after development of the film in a darkroom. Each well was loaded with 10mg protein solution. Each cell line was blotted in triplicates on the same gel. The band of mES-2 was taken as reference band between blots.

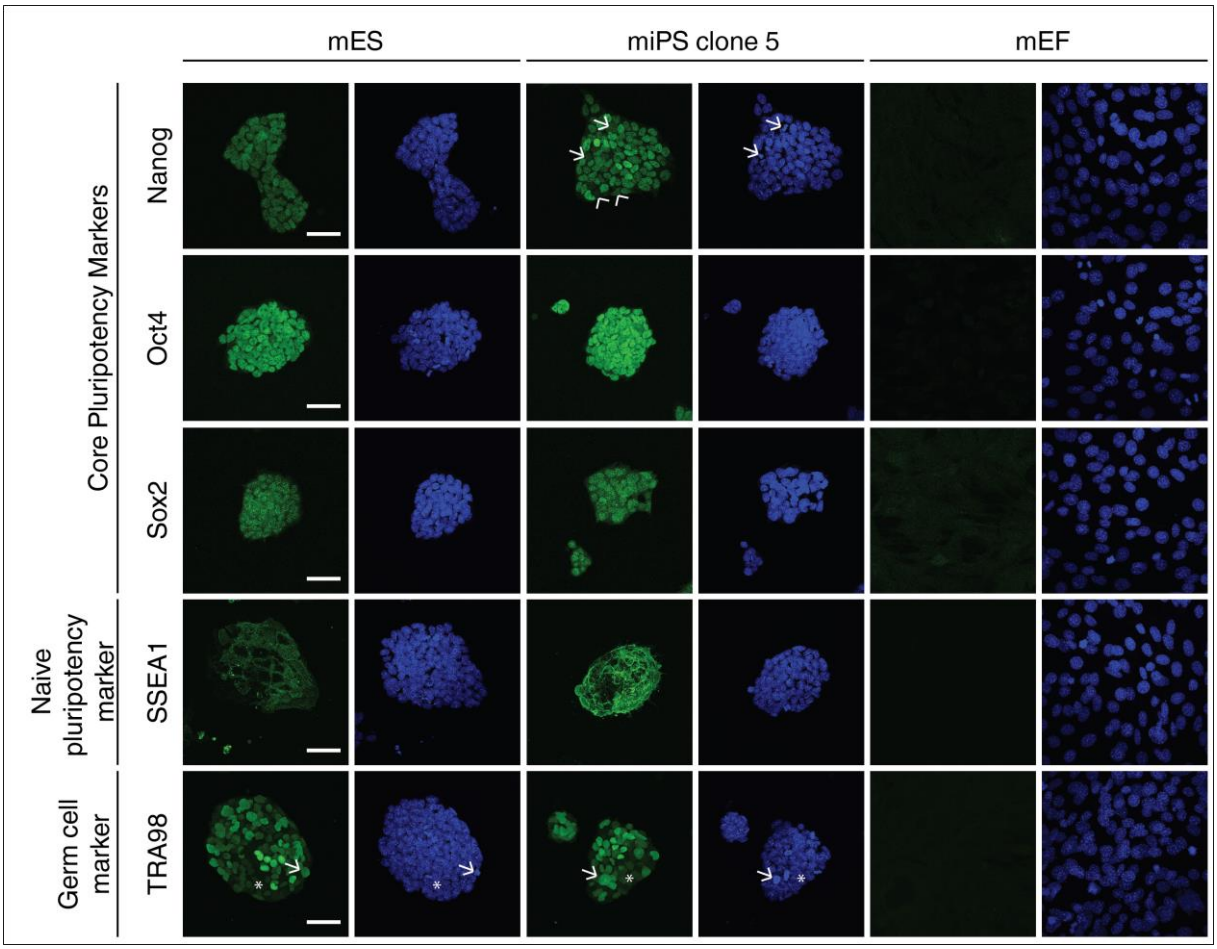


Fig. 4

Immunofluorescence images. All images were taken with a 63x oil UV objective.

Green = epitope, blue = DAPI, scale bars = 50µm

Images of mES, miPS clone 5 and mEF cells showed expression of core pluripotency markers in both mES and miPS clone 5 cells, but not in mEF. mES cells show a more uniform, but weaker signal distribution of Nanog compared to miPS clone 5 cells. In clone 5 the weak signal is in some cases linked to cells during the metaphase (arrows) but independent of the state of the chromatin density as well (arrowheads). The naïve pluripotency marker SSEA1 signal is stronger as well in clone 5 compared to mES cells. The TRA98 stain shows an irregular pattern between high and low-level expression areas in both mES and clone 5. Low signal areas (stars) are

independent of the state of chromatin as well as the cells with high signal for TRA98. mEF cells showed no sign of the stained markers expect for unspecific background staining for Sox2.

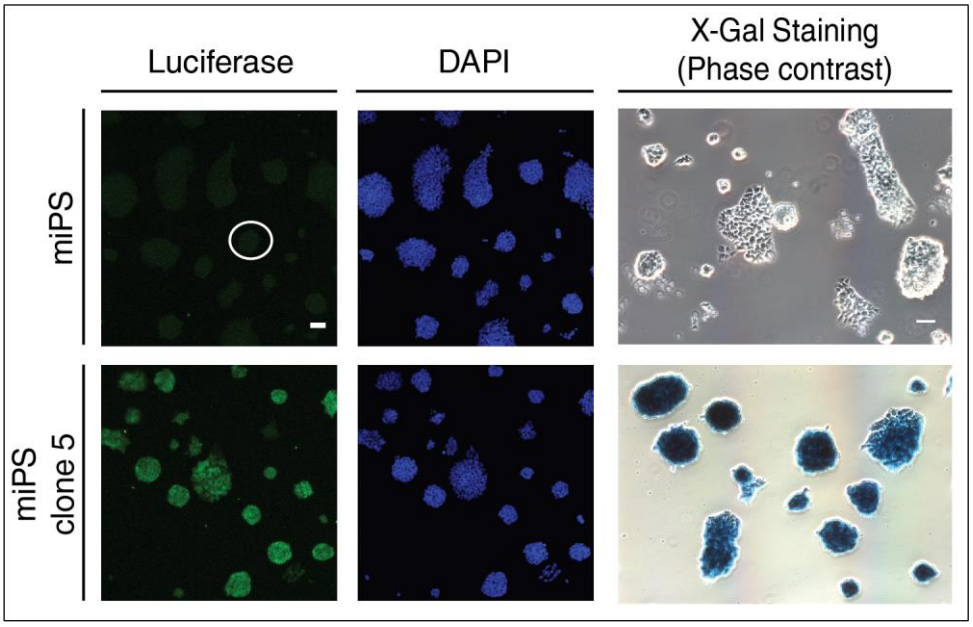


Fig. 5

Confocal immunofluorescence images of luciferase and DAPI images taken with a 20x air objective. Green = epitope, blue = DAPI, Scale bar =50µm

X-Gal staining pictures were taken with a phase-contrast microscope with a 10x air objective. Blue = X-Gal, Scale bar = 50 µm

The native miPS cells showed only a weak background staining (circle). miPS clone 5 cells showed a high signal of luciferase. The blue colonies indicate a high activity of β-Galactosidase in miPS clone 5 cells. Native miPS cells did not show β-Galactosidase activity.

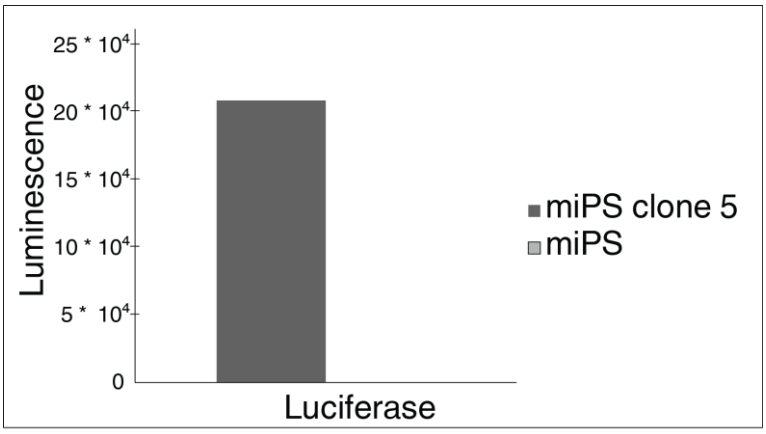


Fig. 6

Luciferase assay of miPS and miPS clone 5 cells. All samples were analyzed in triplicates. This analysis showed a massive signal of luciferase in miPS clone 5 cells compared to miPS cells. In miPS cells the signal was near to non-detectable (mean = 41).

4.2 miPS clone 5 cells show sex-chromosome alterations

Another goal for this study was to identify the cells after they were injected into the mice. To have an additional marker we targeted Y-Chromosome-specific genes. The miPS cells were XY-karyotype male cells and the mice were XX-karyotype females. Since the injected cells originated from male mice and the mice for the teratoma assay were female, a clear distinction between mouse cells and injected transgenic cells should have been possible. We have chosen Sry and Zfy which represent Y-Chromosome-specific genes in mice and Xist as an X-Chromosome-specific gene.

Preliminary real-time PCR results were not able to detect Sry or Zfy in miPS clone 5 cells. We therefore extended the analysis to more cell lines, to rule out eventual technical errors. The analysis of five cell lines revealed that the transgenic cell lines mES clone 11, miPS Luc/Lac and miPS clone 5 showed a signal for the transgenes luciferase and LacZ but not for Sry and Zfy (Fig. 7). In the original cells that were purchased (mES, miPS, miPS Luc/Lac), Zfy and Sry were detectable.

For further investigation of the chromosome set we decided to conduct fluorescent in-situ hybridization (FISH) analysis of the whole chromosome set of miPS, mES and miPS clone 5 cells. The analysis confirmed the previous PCR results. While the original miPS and mES cells showed a signal for the Y-Chromosome, the miPS clone 5 cells did not show a signal (Fig. 8A). In miPS clone 5 only a small band appeared inside the telomere region of the X-Chromosome. A karyogram of the miPS clone 5 cells showed that there are no further chromosome set alterations in terms of polyploidity or lack of another chromosome (Fig. 8B).

These results have a high impact on this study, since chromosome alterations would prohibit the application of cells in clinical trials for fertility restoration. Furthermore the question was raised if male cells without Y-Chromosome can give rise to healthy and fully functional male germ cells *in vivo*. The results also implicated a more elaborate and thoroughly screening of cells during single-cell clone isolation in the future.

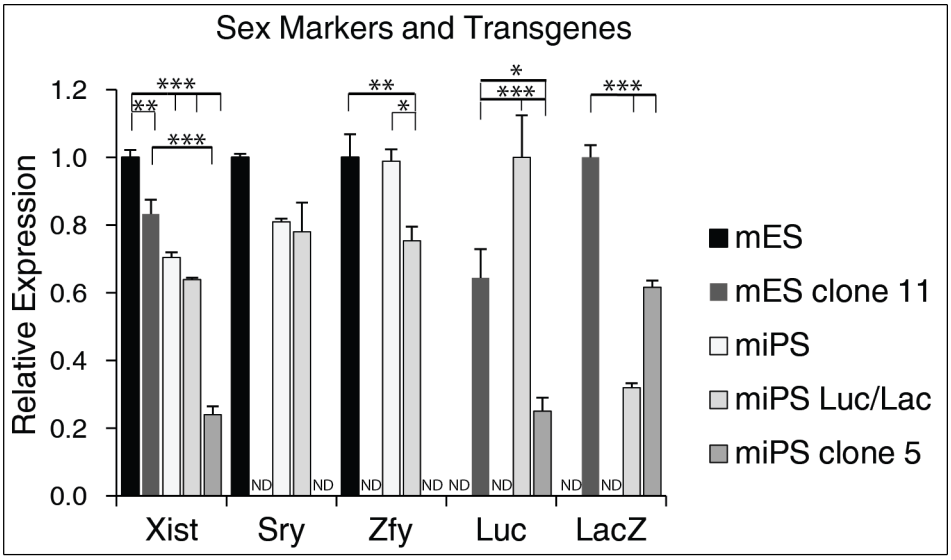


Fig. 7

Real-time PCR of DNA of mES and miPS cells. Erros bars correspond to the S.E.M. Data were generated from duplicates of three independent experiments. All cells originate from XY-karyotype murine cells. One star represents $p<0.05$, two stars represent $p< 0.01$, three stars represent $p<0.001$. miPS clone 5 is a single cell-derived cell clone of miPS Luc/Lac cells.

Xist is detectable in all five cell lines. mES cells showed a significant higher signal compared to the other cell lines for Xist, Zfy and Sry. Signals for Sry and Zfy were not detectable in miPS clone 5 and mES clone 11, but in mES, miPS and miPS Luc/Lac cells respectively. The signal of luciferase was significantly higher in miPS Luc/Lac cells than compared to miPS clones 5 and mES clone 11. Differences between the miPS clone 5 and mES clone 11 showed low significance. mES clone 11 showed the highest signal for LacZ compared to the transgene cell lines miPS Luc/Lac and miPS clone 5.

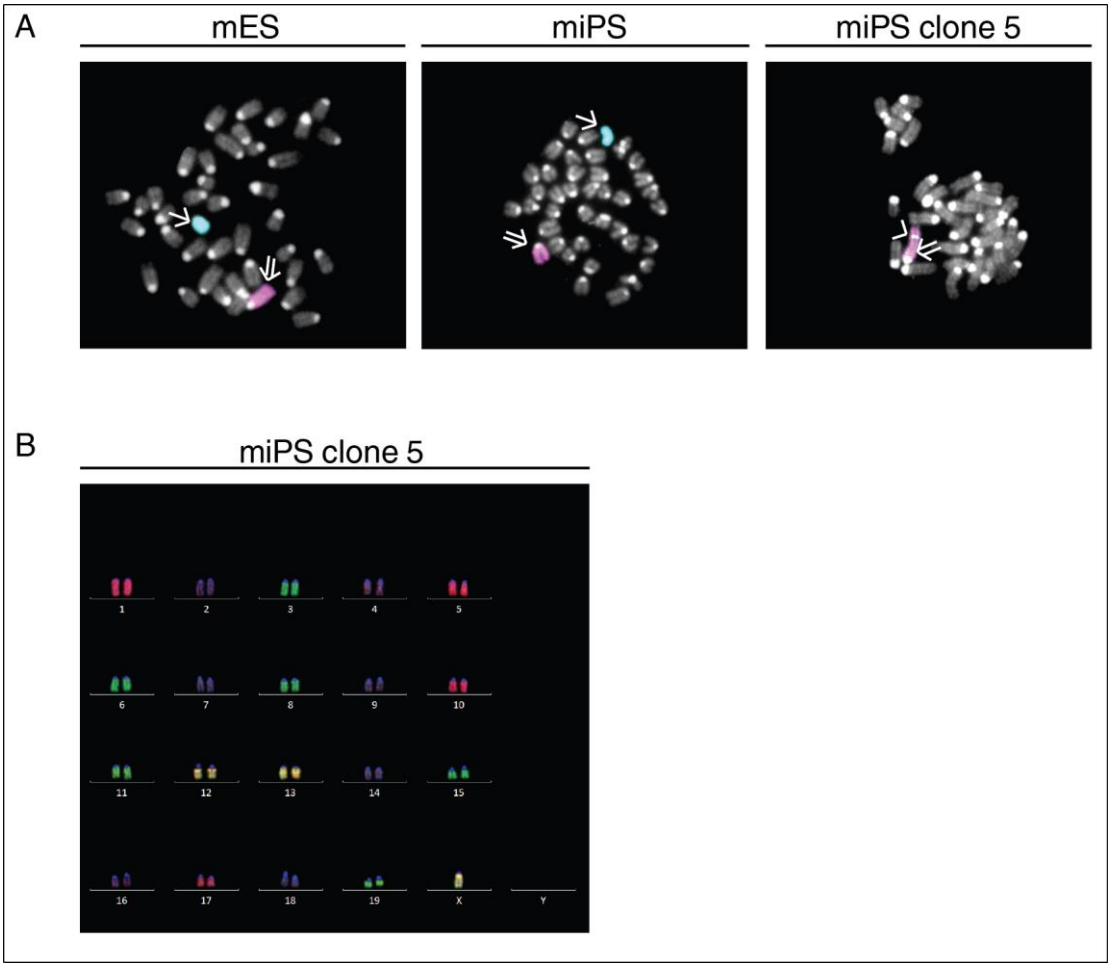


Fig. 8

(**A**) FISH painting of Chromosomes. Pink = gonosomal X-Chromosome, blue= Y-Chromosome

(**B)** Complete karyogram of miPS clone 5 cells

FISH painting and karyogram of miPS clone 5 cells revealed that miPS and mES cells have very well visible sex chromosomes (pink staining, double arrow = X-Chromosome and blue staining, arrow = Y-Chromosome). miPS clone 5 cells showed a small band inside the arms of the sex-Chromosome (pink staining, arrowhead). The band appears in the distal region of the X-Chromosome (arrowhead) and the X-Chromosome seems elongated. The chromosome analysis confirmed the absence of a Y-chromosome.

4.3 An extended unstimulated time period of GoST cells reveals a new cell phenotype

One of the major questions addressed by this study was if GoST induction can be conducted with miPS cells in changed culturing methods by addition of 2iL to the medium.

miPS clone 5 cells were cultured for 10 passages in 2iL + βME plus Ex527 and RG108. The resulting cells were described as cmiPS clone 5 cells. WT cells were cultured in 2iL + βME + DMSO. cmiPS clone 5 cells were deprived of 2iL and tBHQ was added for seven days. WT cells were deprived of 2iL as well and were cultured in BM with DMSO. After seven days the

cmiPS clone 5 cells showed the morphology of GoST cells[1] and appeared vital (Fig.9). The resulting cells were described as GoST cells. Flat cells grew to 100% confluence between the former colonies (Fig. 9). In WT cells the colonies appeared as dark spots and small round cells were floating between the colonies (Fig. 9).

After this preliminary experiment we conducted the GoST induction on cmiPS clone 5 cells and compared the macroscopic appearance and mRNA expression patterns of pluripotency, germ cell and spermatogonial stem cell (SSC) markers to WT treated cells. After GoST induction of cmiPS clone 5 cells we observed both groups for an unstimulated time period of 20 days in BM plus DMSO. For WT cells the observed time points were taken before the unstimulated time period (D0) and in four-day intervals (D0+4, D0+8, D0+12, D0+16, D0+20). For cmiPS clone 5 and GoST cells the observed time points were after 10 passages in conditioning medium, right before GoST induction (D0), right after GoST induction (D7) and then in four-day intervals (D7+4, D7+8, D7+12, D7+16, D7+20). The cells after D7 were described as GoST cells.

Light microscopy images show that the morphology of the cells does differ in terms of cell-layer thickness and height of the former colonies until 12 days without stimulation (Fig. 10) between WT and GoST cells. WT cells did show a thicker cell layer between the colonies whereas GoST cells appeared to have only a thin layer of cells. Both groups showed black spots were the former cell colonies were. After this point WT cells did not show a significant change to their morphology (Fig. 10). After 16 days without stimulation GoST cells spawned small round cells around and on top of former colonies that were multiplying until D7+20 and filled the dish (Fig. 10).

This unexpected observation raised the question if the round cells resemble a known cell type. Since the GoST induction induces germ cell markers we suspected the round cells to be germ cell- or SSC-like cells.

For further investigation of the GoST and round cells, real-time RT-PCR of mRNA from GoST and WT cells was conducted.

At first, we investigated core and naïve pluripotency markers throughout the observed time points. Especially with regard to the previous observation of the round cells we were curious how the pluripotency develops after D7+16 and if we can detect signs of differentiation into SSC-like cells.

Most of the core pluripotency markers were significantly downregulated during the unstimulated time period (Fig. 11). Only *Klf4* and *Tbx3* were still expressed after release of 2iL. In GoST cells the expression of *Klf4* showed a high increase after D7. WT cells did express *Klf4* as well, but in lower fashion compared to GoST cells. Both groups showed initial downregulation of *Klf4* expression, but then showed upregulation (Fig. 11). In WT cells the expression of *Klf4* peaks at D0+12 and decays afterwards (Fig. 11). GoST cells show a high increase of *Klf4* expression from D7 until D7+20 with significantly higher expression compared to WT cells (Fig. 11). The *Tbx3* expression showed no significant differences between the two groups in the observed time points (Fig. 11). Expression of *Tbx3* in WT cells peaks at D0+12 and then decreases until D0+20 (Fig. 11). *Tbx3* expression in GoST cells peaks at D7+4 and D7+8 and then decreases (Fig.11). In previous work using mES cells[1], it was shown that GoST cells maintain expression of core pluripotency markers at similar levels compared to untreated cells at D0. Contrary to this previous work[1], the expression of all core pluripotency markers (*Nanog*, *Oct4*, *Sox2*, *Nr5a2*, *Esrrb*, *Klf4*, *Tbx3*) was downregulated in GoST cells (D7) compared to WT cells (D0). This observation implies that miPS clone 5 cells in the current study did not react in the same way to GoST induction as mES cells in previous work[1].

Analysis of naïve pluripotency markers showed downregulation in both groups throughout the unstimulated time period.

Dax1, *Dppa3* and *Prdm14* were highly downregulated after 20 days of unstimulated culturing (Fig. 12). *Fbxo15* was highly downregulated after 20 days too, but increased shortly at D0+12 in WT and D7+20 in GoST cells (Fig. 12). *Prdm14* showed similar expression patterns in WT and GoST cell. Expression levels of *Piwil2*, *Rex1* and *Stra8* stand out in this analysis. *Piwil2* expression increased in both groups between 4 and 12 days in unstimulated culture conditions (Fig. 12). *Rex1* shows a plateau phase after an initial decrease of expression at D0. After 12 days of unstimulated culture conditions the expression decreases slightly in the next observed time points. *Stra8* shows low levels of expression in both groups initially. In WT cells *Stra8* expression increases after 8 days in unstimulated culture conditions with a peak at D0+12. Afterwards the expression is heavily downregulated to levels equal to D0. In GoST cells *Stra8* expression levels show a high increase after 4 days of unstimulated culture and peaks at D7+8

(Fig.12). Afterwards *Stra8* expression is massively downregulated to near to non-detectable levels at D7+20 (Fig. 12).

The analysis of core and naïve pluripotency markers revealed that both WT and GoST cells are no longer pluripotent since most of the markers were downregulated during the unstimulated time period (Fig. 11 and 12). This indicates that WT and GoST cells have differentiated. Most of the expression patterns throughout the observed time series did not differ between WT and GoST cells. Since GoST induction induces germ cell-like features in mES cells, our next step was the investigation of germ- and spermatogonial stem cell-specific markers on mRNA levels.

The analysis of germ cell markers showed that most of the markers were detectable in both groups at some points of the observed time points (Fig. 13). An interesting observation was that some markers showed similar expression patterns in both groups. *Ddx4* shows an increase of expression until 12 days in unstimulated culture conditions and then gets downregulated until 20 days (Fig. 13). *Nanos2* is expressed at low levels in the first two time points and then gets highly upregulated after 8 and 12 days (Fig. 13). At 16 and 20 days, *Nanos2* gets downregulated to basal levels. *Dazl* expression is upregulated in both groups as well, but the peak is shifted. In WT cell the peak expression of *Dazl* is detected at D0+12 and then expression decreases massively. In GoST cells the peak expression of *Dazl* can be detected after 8 days in unstimulated culture conditions and then decreases massively. WT cells did also show a plateau phase of *Dazl* expression from D0 to D0+8. *Zbtb16* expression peaks at the same time points as *Dazl* in both groups and then the expression decreases. A prominent difference between both groups was the very low expression of *Zbtb16* in WT cells at D0 and D0+4 compared to D7 and D7+4 in GoST cells. *Tdrd1*, *Plk1s1* and *Tex101* showed upregulation throughout the observed time points in both groups. *Tex101* is highly upregulated in WT cells from D0+4 until D0+12 and then downregulated to a plateau level at D0+16 and D0+20. GoST cells show a peak in *Tex101* expression at D7+4 and then expression is downregulated to levels of D7 at time points D7+16 and D7+20. *Plk1s1* expression peaks around 12 days in unstimulated culture conditions in both groups. The overall expression of *Plk1s1* is higher in GoST cells compared to WT cells and peaks in both groups after 12 days in unstimulated culture conditions. Expression of *Tdrd1* decreases in both groups at 4 (GoST cells) and 8 days (WT cells) in unstimulated culture conditions. The peak expression of *Tdrd1* is at D0+16 in WT cells and D7+8 in GoST cells. After the peak,

Tdrd1 is downregulated in both groups. The overall expression of *Tdrd1* is higher in WT cells compared to GoST cells.

Analysis of germ cell markers showed that in both groups deprivation of inhibiting factors induces germ cell-specific markers. Based on our observations the most important period of time seems to be around 8 to 12 days after release from 2iL. Especially the similarities in expression patterns throughout the observed time points stand out compared to the core and naïve pluripotency markers. The round cells appeared 16 days after release from 2iL while most of the germ cell markers are downregulated after this time point. Thus a clear correlation between the analyzed germ cell markers and the appearance of the round cells could not be found.

Due to the morphology of the round cells which, showed similarities to SSCs, we investigated SSC-specific markers as well. While most of the SSC markers are not expressed at D0/D7 in both groups, the markers were upregulated during the observed time period (Fig. 14). *CD53*, *Csf1r* and *Ifi203* were not or extremely low expressed in both groups at D0/D7. All three markers showed upregulation after the release of 2iL. Upregulation of *CD53* only appeared at D0+16 and D0+20 in WT cells. In GoST cells *CD53* was upregulated 8 days after release and peaked at D7+12. Then the signal decayed until D7+20 in GoST cells. The expression of *CD53* was significantly higher in GoST cells compared to WT cells. *Csf1r* showed similar expression patterns, although the expression started earlier in both groups (Fig.14). In WT cells *Csf1r* expression started 4 days after release of 2iL and continuously increased over time. GoST cells already expressed *Csf1r* at very low levels before release at D7. The expression peaks at D7+16 and then decreases in GoST cells. The overall expression of *Csf1r* is significantly higher in GoST cells than in WT cells. Exactly the same expression pattern was observed for *Ifi203*, with a constant increase of expression in WT from D0+8 on and a peak expression at D7+16 in GoST cells (Fig.14). *Col1a2* and *Gfra1* did not show such a regular expression pattern in both groups compared to the other markers. *Col1a2* showed upregulation on WT cells from D0 until D0+8. Then the signal decreases until D0+16 and increases again at D0+20. In GoST cells the expression of *Col1a2* showed a steady decrease from D7 until D7+12. Then the *Col1a2* gets upregulated at D7+16 and downregulated at D7+20. *Gfra1* showed a peak expression in WT cells at D0+4 and then decreases until D0+20. GoST cells showed a steady upregulation of *Gfra1* throughout the observed 20 days. The peak at D7+20 was significantly higher compared to the peak at D0+4 of WT cells.

The results of SSC markers showed upregulation of the tested markers in both groups. The peak expression points were generally higher in GoST cells except for *Col1a2*. A striking result is that SSC markers were expressed in both groups. This result was unexpected, since usually PS cells tend to differentiate into neuroectodermal cells when released of inhibiting factors. The only SSC marker that shows upregulation in correlation with the appearance of the round cells is *Gfra1* with a peak expression at D7+20 in GoST cells and low levels of expression in WT cells. We therefore expected that *Gfra1* upregulation is a result of the growing round cell population, since they only appeared in the GoST cell group. Nevertheless the 16-day mark after release of 2iL seems to be an important time point in terms of SSC marker expression, since in both groups the expression of most of the markers either increased or decreased massively.

Since Gfra1 seemed to be the only marker of which the expression fits the appearance of the round cells we decided to conduct confocal immunofluorescence images with anti-Gfra1 antibodies (Fig. 15). As we assumed that the round cells resemble SSC-like cells, we additionally stained for the germ cell-specific marker TRA98 (Fig. 15 and 16).

The confocal images showed that the expression of TRA98 is not only found in GoST cells, but in WT cells as well (Fig. 15 and 16). The round cells showed a weak signal for TRA98 at D7+16 and D7+20 while WT cells showed a more intense staining compared to the round cells throughout the observed time points (Fig. 15 and 16). Gfra1 showed unspecific background staining and no correlation between signal and round cells in GoST cells or WT cells (Fig.15).

The confocal images showed no clear evidence of SSC-like cellular features of the round cells. Since TRA98 and Gfra1 showed no distinct difference between the two groups, the question which kind of cells the round cells represent remained unclear.

Overall the characterization of the GoST induction series revealed that GoST and WT cells lose pluripotency and germ cell and SSC markers are induced after the release of 2iL.

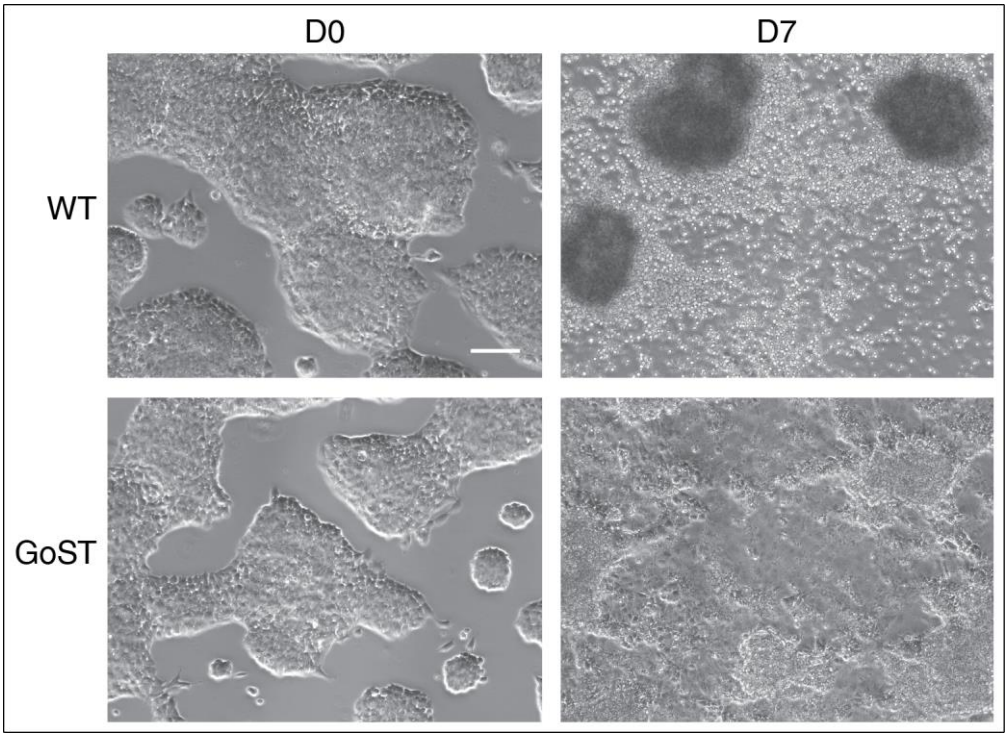


Fig. 9

Phase-contrast microscopy of miPS clone 5 cells with and without GoST induction after seven days. Images were taken with a 10x air objective. Scale bar = 100 µm

These images show the effect of the GoST induction in comparison to exposure to BM and DMSO. WT and cmiPS clone 5 cells look vital and similar in shape and thickness of the colonies at D0. In GoST cells, after seven days of release from 2iL plus βME, the area between the former colonies is filled with flat cells. In WT cells the space between the former colonies is filled with cellular debris. Cells in the WT–treatment did float around and could be washed away easily; therefore this sample was not mounted with a coverslip. Furthermore, WT cells appear to be dead and are scattered around the former colonies at D7. GoST cells appeared vital and were able to fill the gaps between the colonies with flat cells. GoST cells were sticking tightly to the surface of the culture dish and could neither be washed away nor dissociated with Accutase.

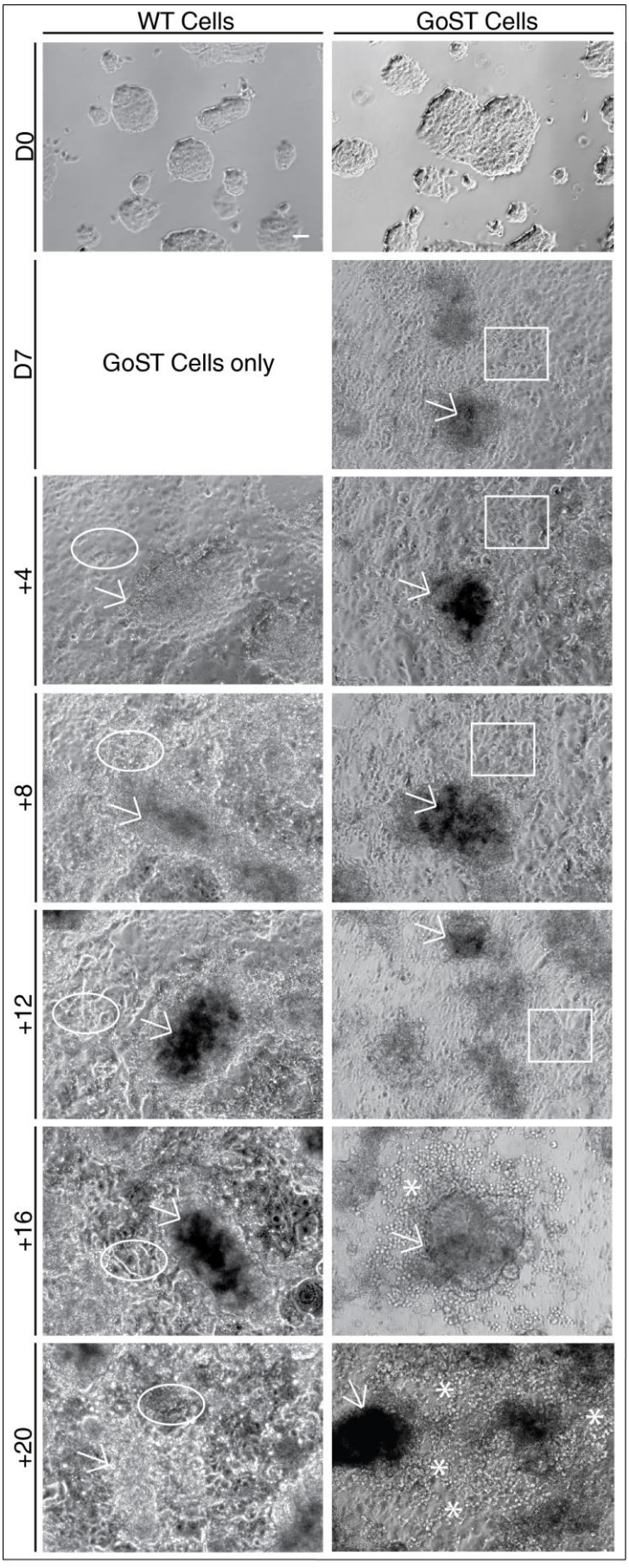

WT Cells
GoST Cells
D0
D7
GoST Cells only
+4
+8
+12
+16
+20

Fig. 10

Phase contrast microscopy of WT and GoST cells after release of 2iL medium. Images were taken with a 10x air objective. Scale bar =50 µm

The comparison of WT and GoST cells from D0 or D7 on respectively until 20 days without stimulation revealed differences in cell morpholgy. Differences lie in the thickness of the cell layer between the colonies. Initially there are no cells between colonies in both groups. The colonies undergo a change in appearance from translucent at D0 to dark and opaque during the series (arrows). This effect appears earlier in GoST cells (D7) compared to WT cells (+12). For GoST cells, this effect decreases after +12. At time point D0+4 for WT and D7 for GoST cells, a thin layer of cells (circle for WT cells, square for GoST cells) grew between the colonies (arrows). While the WT cells develop a thick cell layer from timepoint +8 on, the GoST cells have a thin cell layer in this region until +16. The layer in WT cells does not change significantly after time point +12, whereas in GoST cells small round cells appear in the periphery of former cell colonies (star). Those cells appeared in higher amounts at time point +20, predominating the field of view in the culture dish and overgrew the underlying flat cell-layer at this point. The round cells could be found floating in the culture medium. Round cells did not appear in WT cells at any observed time point.

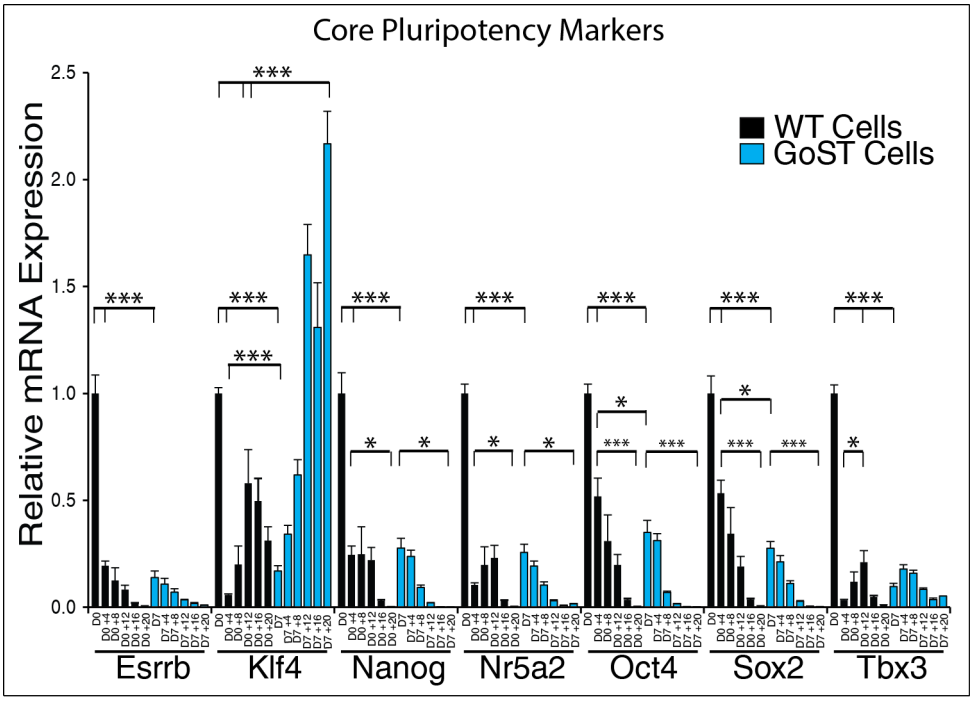


Fig. 11

Real-time RT-PCR of mRNA for core pluripotency markers of the GoST series, normalized against Glycerine-3-Phosphate-Dehydrogenase (Gapdh). Data were generated from duplicates of three independent experiments. The error bars correspond to the S.E.M. One star represents $p<0.05$, two stars represent $p<0.01$, three stars represent $p<0.001$.

The investigation of the mRNA expression of core pluripotency markers during GoST induction or WT-treatment showed an overal downregulation except for Klf4. Klf4 showed upregulation in GoST cells. First,

there is a highly significant decrease in expression in both groups, but over time Klf4 is higly upregulated in GoST cells until timepoint D7+20. In WT-cells the increase peaks at timepoint D0+12 and then decreases. The expression of Klf4 at timepoint D7+4 and D7+20 is significantly higher in GoST cells compared to WT cells. Expression of Nanog, Sox2, Oct4 and Esrrb decreases over the observed time points to near to non-detectable levels. The Tbx3 expression throughout the unstimulated time period showed a significant increase in the WT cells from timepoint D0+4 to D0+12, then the signal decreased again near to non detecable levels. In GoST cells no significant change from D7 on was observed despite the initial decrease in expression for Tbx3.

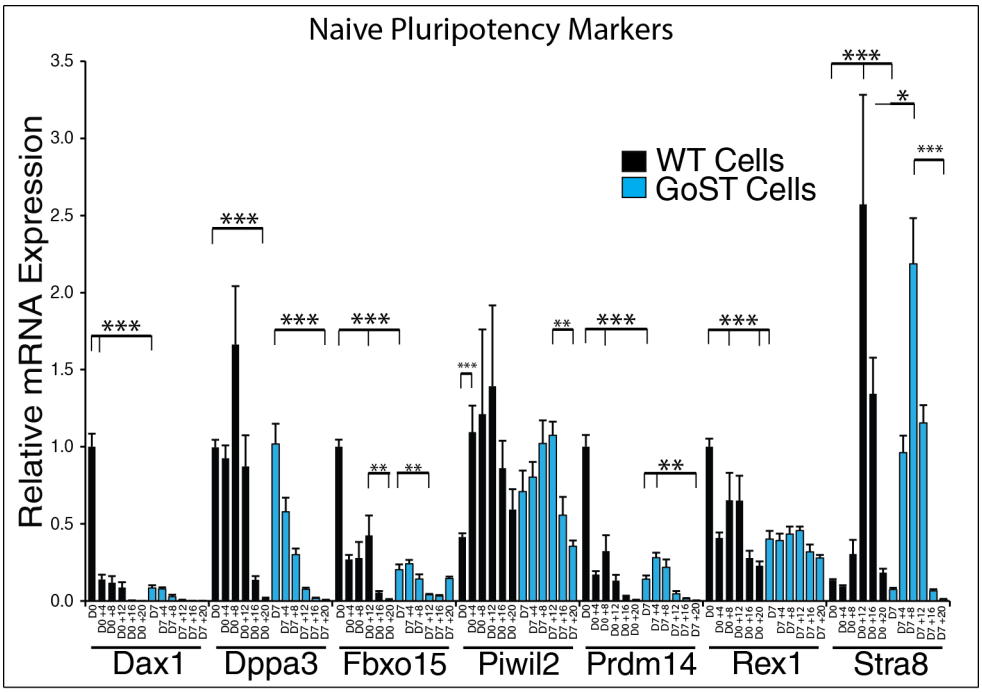


Fig. 12

Real-time RT-PCR of mRNA for naïve pluripotency markers of the GoST series, normalized against Gapdh. Data were generated from duplicates of three independent experiments. The error bars correspond to the SEM. One star represents $p<0.05$, two stars represent $p<0.01$, three stars represent $p<0.001$.

The analysis of naïve pluripotency markers of the GoST series revealed that all markers except for Piwil2 and Rex1 decreased over time. Dax1, Dppa3, Fbxo15 and Prdm14 show significant downregulation over the 20 days of unstimulated culture. Dppa3 showed a plateau phase from D0 until D0+12 and then decreased expression until D0+20 in WT cells. In GoST cells a highly significant and steady decrease of Dppa3 from D7 until D7+20 appears. Fbxo15 expression increases at time point D0+12 after a plateau phase until time point D0+8, but then decreases in the last two time points. Rex 1 shows a highly significant initial decrease and plateaus throughout the whole unstimulated time period in both groups. Expression levels of Stra8 showed in both groups a high increase until D0+12 or D7+8 respectively, while both peaks differ significantly in signal. After this the peak of Stra8 expression decreases until the last observed time point. Piwil2 shows the same pattern of increasing signal in WT and GoST cells. Piwil2 expression increases until 12 days under unstimulated culture and then decreases to equal levels of xpression in WT cells at time point D0.

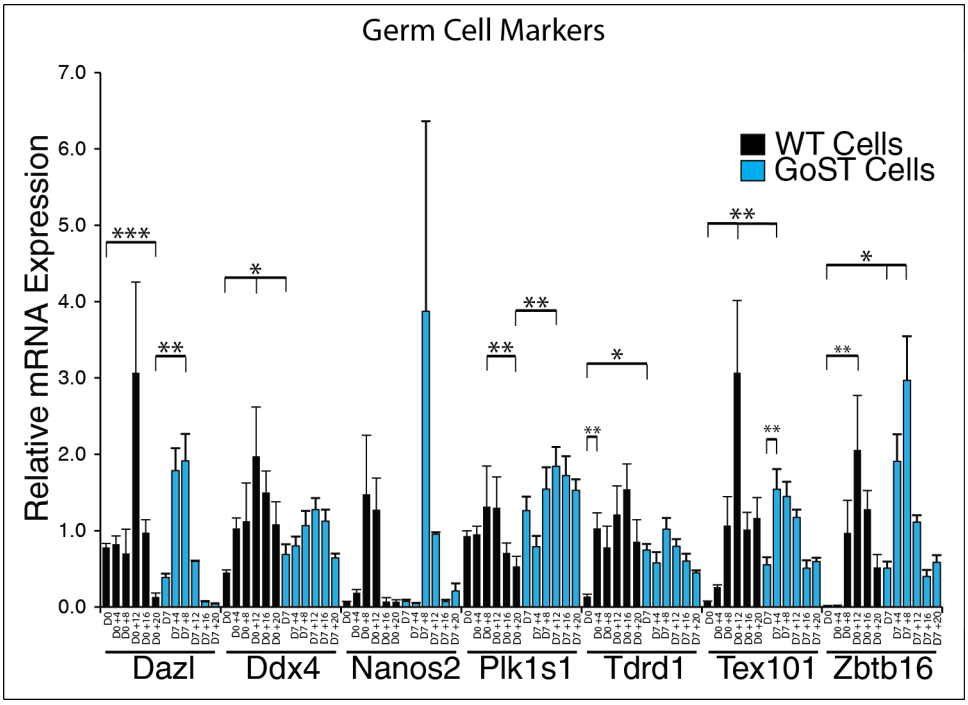


Fig. 13

Real-time RT-PCR of mRNA for germ cell markers of the GoST series, normalized against Gapdh. Data were generated from duplicates of three independent experiments. The error bars correspond to the SEM. One star represents $p<0.05$, two stars represent $p<0.01$, three stars represent $p<0.001$.

Analysis of mRNA expression levels shows a highly significant decrease of Dazl expression after an increase in WT cells until day 16 under unstimulated culture conditions. In GoST cells the Dazl expression increases at D7+4 until D7+8, but decreases as well from time point D7+16 on. The expression patterns in WT and GoST for Ddx4 show similarities. In WT cells the increase of expression is significantly higher than in GoST cells although expression at D7 in GoST cells is already significantly higher than in WT cells. Although the peak is not significantly higher than the start and the end points, an increase of signal at the D7+12 is detectable which decreases off until D7+20, just like in WT cells. The expression of Nanos2 only shows high expression at time points +8 and +12 in WT and GoST cells while GoST cells showed a higher expression but with a very high SEM value. Plk1s1 and Zbtb16 show similar patterns in the two groups. First, there is an increase of expression until 12 days in unstimulated culture conditions. Afterwards a significant decrease to almost starting levels is present in both WT and GoST cells. The overall expression of Plk1s1 and Zbtb16 is higher in GoST cells compared to WT cells. The expression of Tdrd1 is higher in WT cells throughout the series compared to GoST cells, although the first time points are significantly higher in GoST cells. After the increase of expression af Tdrd1 until 12 or 16 days in unstimulated culture conditions the expression decreases to starting levels. For Tex101 the peak expression is significantly higher in WT cells than in GoST cells, but decreases after time point +12. In GoST cells the expression levels to the D7 time point, while in WT cells the increase of expression 20 days after release of 2iL is significantly higher than at D0.

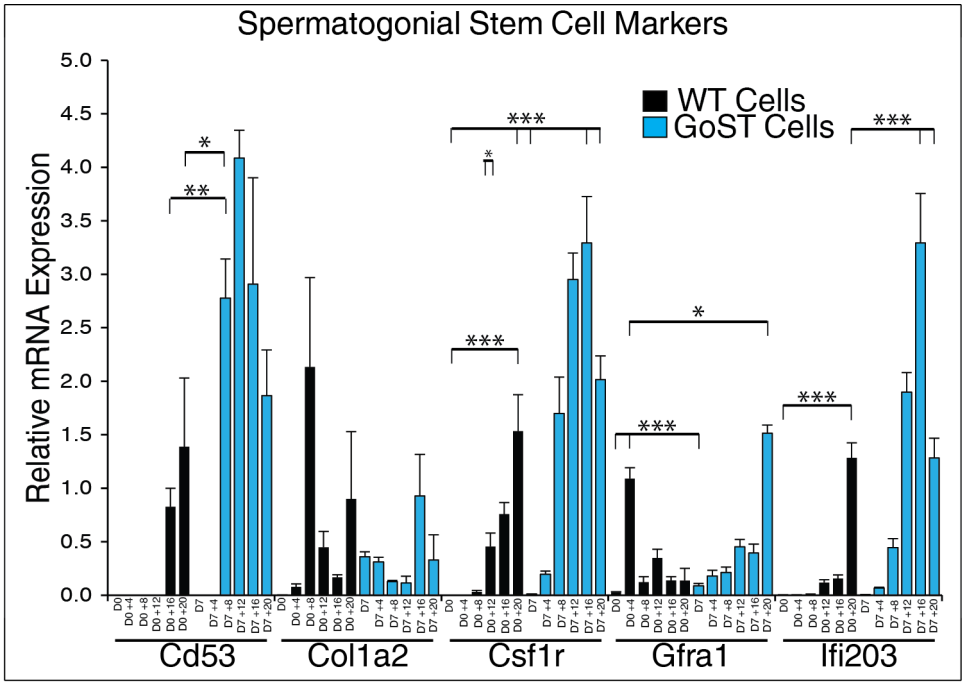


Fig. 14

Real-time RT-PCR of mRNA for spermatogonial stem cell markers of the GoST series, normalized against Gapdh. Data were generated from duplicates of three independent experiments. All samples were analyzed in triplicates. The error bars correspond to the S.E.M. One star represents $p<0.05$, two stars represent $p<0.01$, three stars represent $p<0.001$.

At first, some spermatogonial stem cell markers were not expressed in both groups, but showed significant differences throughout the series. In Cd53 the expression started in WT cells at D0+16, but did not increase significantly at D0+20. In GoST cells the expression starts at time point +8, peaks at +12 and decreases until 20 days after release of 2iL. The expression of Cd53 is significantly higher in GoST cells compared to WT cells until 16 days after release of 2iL. The Csf1r expression shows a highly significant increase in both WT and GoST cells. In GoST cells Csf1r expression is higher compared to WT cells and decreases after the peak at time point +16. This peak shows a high significance towards the other time points with an approximate 2 fold higher expression. The expression of Gfra1 shows a great difference between the two groups. In WT cells the expression increases from timepoint D0 to D0+4, but decreases afterwards and stays at this level throughout the remaining time points. In GoST cells Gfra1 expression increases steadily throughout the series, but also shows significantly higher levels of expression at its peak at +20 compared to WT cells at time point D0+4. Althought the level of expression in GoST cells does not increase significantly until D7+16, at D7+20 the expression is massively increased.The expression of Ifi203 starts earlier in GoST cells and peaks at time point D7+16, while in WT cells the expression is delayed to D0+12 and peaks at D0+20. The peak expression of Ifi203 is significantly higher in the GoST cells compared to WT cells. 20 days after release of 2iL the expression of Ifi203 does not differ significantly between both groups.

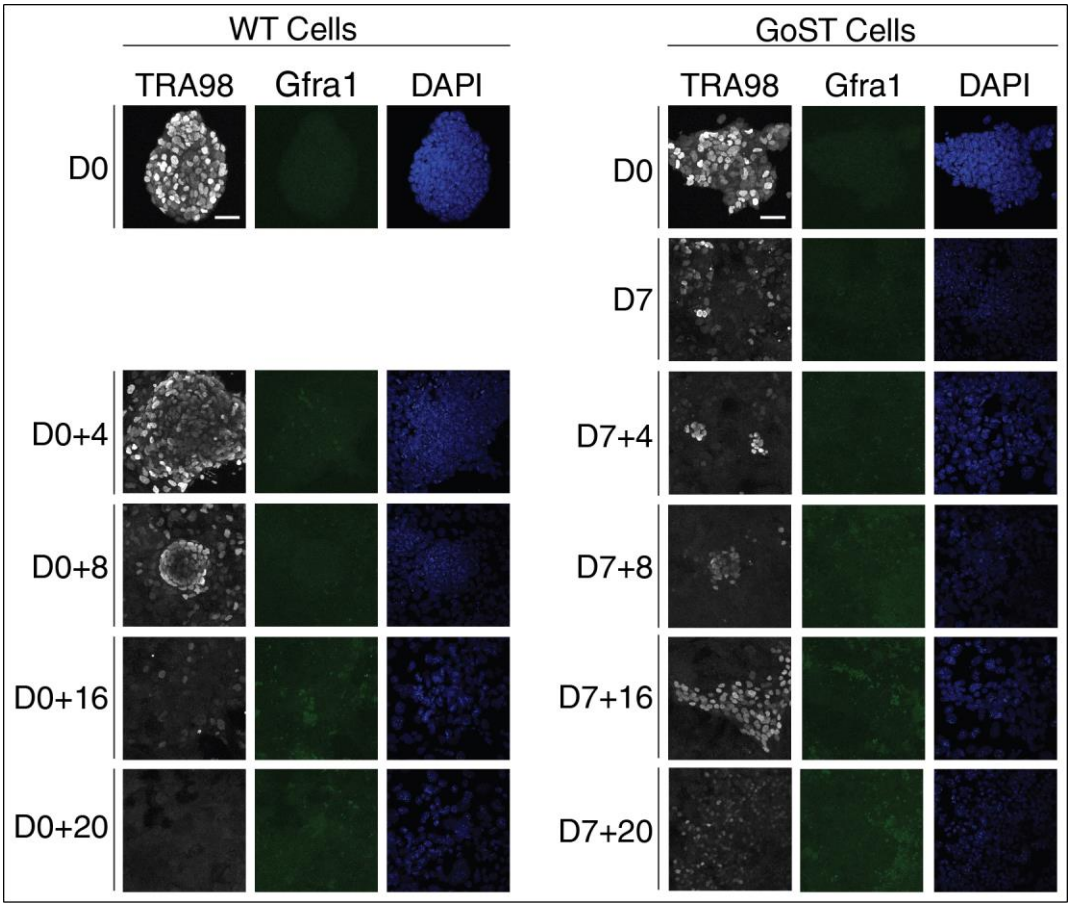


Fig. 15

Immunofluorescence images of the GoST series. All images were taken with a 63x oil UV objective.
Greyscale = TRA98, Green = Gfra1, Blue = DAPI, Scale bars = 50µm

These images show the expression of the nuclear marker TRA98 and the surface marker Gfra1. At D0 the TRA98 stain shows the previously observed heterogeneous pattern in both WT and GoST cells, before the start of the chemical treatment. While the TRA98 signal decreases in WT cells over the observed time points, the GoST cells still show positive cells until 20 days after release of 2iL. The image at D7+20 in GoST cells shows a cell cluster of round cells. These cells still have a TRA98 signal, although it is weaker than in colonies at D7+16. The Gfra1 signal in general shows heterogeneous staining patterns. This signal is weak to non-detectable in WT cells over the 20 days. The only signals are detectable at D0+16 and D0+20 in a few areas. In GoST cells the signal increases from D7+4 on and seems to be correlated to TRA98-negative cells. At time point D7+20 the Gfra1 positive regions are beneath the TRA98 positive round cells. However the Gfra1 signals does not correlate with the DAPI stain very well. For a cell-surface marker it would be expected to get a similar staining as in SSEA1 (Fig. 4). The DAPI signal shows a high amount of nuclear fragments in the beginning of the unstimulated time period of both WT and GoST cells. These fragments disappear in the course of the unstimulated time period. The nuclei show a high variance in size and shape throughout the series. This does not however correlate with either the TRA98 or Gfra1 signal.

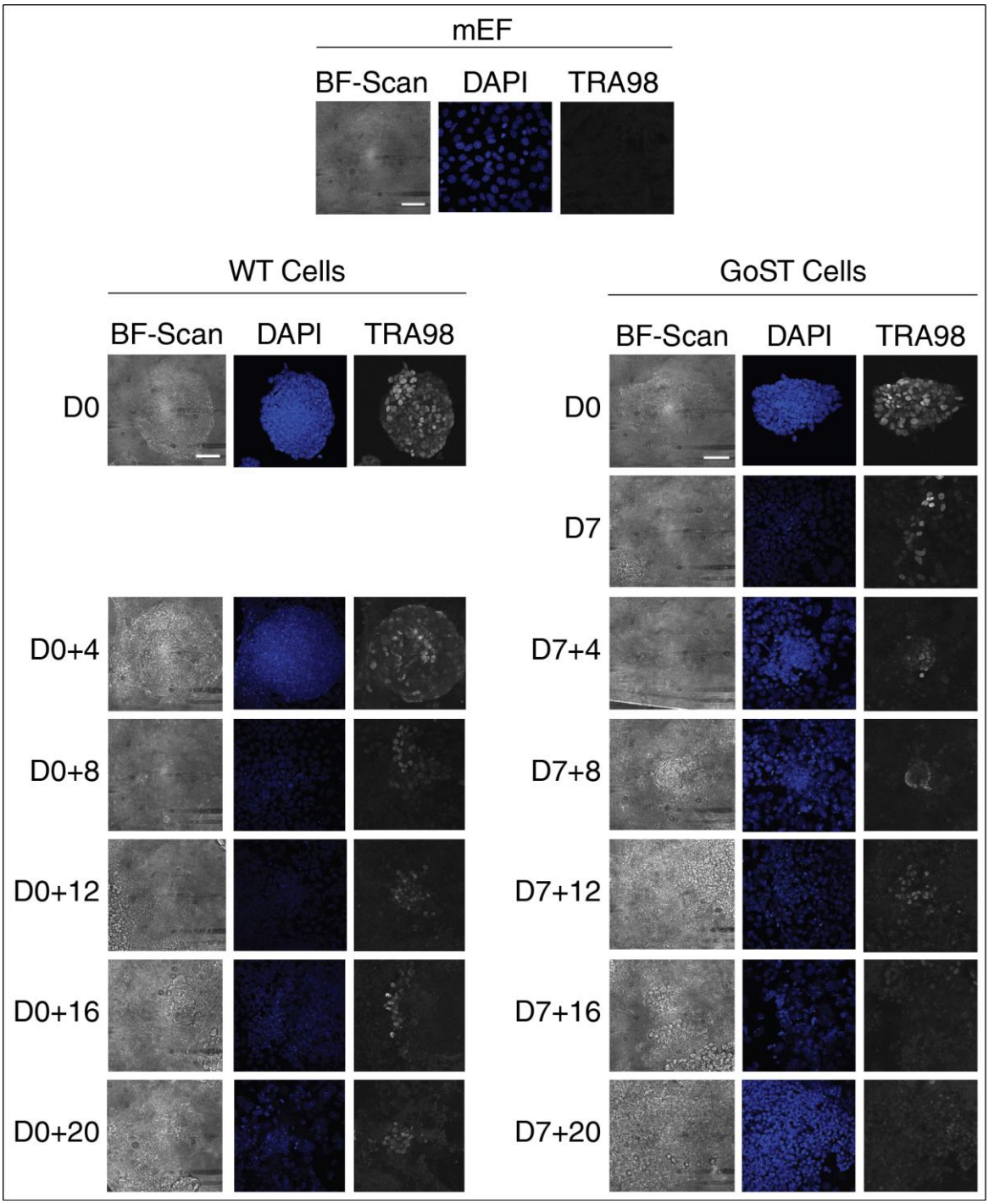


Fig. 16

Immunofluorescence images of the GoST series. All images were taken with a 63x oil UV objective.

Greyscale = TRA98, Blue = DAPI, Scale bars = 50µm

The confocal images of TRA98 antibodies showed that TRA98 is expressed in WT and GoST cells during the 20 days of unstimulated culture. The signal in WT cells is brighter compared to the round cells of D7+16 and D7+20. The round cells expressed TRA98 in irregular patterns throughout the round cell population. mEF cells did not show any sign of TRA98 expression.

4.4 GoST cells show reduced tumor growth, but still contribute to teratoma formation

The measurements of the tumor diameters revealed that in the lowest cell dose no differences between the two groups are visible (Fig. 17). In the 10^4 cell dosage WT cells proliferated in all 5 mice almost uniform and all mice reached the end point at the same time (Fig. 17). In the GoST cell group only two mice reached the end point at the same time as the WT cell injected mice. The remaining mice in this group developed slow growing tumors. Two mice of the GoST cell group reached the end point of the study after 91 days. In the highest cell dosage of $3*10^6$ cells the WT cells proliferated faster and 4 out of 5 mice reached the end point around 27 days of the study (Fig. 17). At the highest cell dosage in GoST cell injected mice the tumors did grew slower than in the WT group and one mouse reached the end point of the study after 91 days (Fig. 17). The analysis of the average survival time (AST) showed that mice that were injected with GoST cells in the two highest cell dosages reached the end point later than mice that were injected with WT cells (Fig.17).

Although GoST cells still contributed to tumor formation, the GoST cell-derived tumors proliferated slower on average compared to WT cells. This effect was positively correlated with higher cell dosage in WT cells. The reasons why GoST cells proliferated slower compared to WT cells and what kind of tumor GoST cell contribute to could not be assessed by this experiment. Therefore we conducted an *in vivo* imaging (IVIS) for luciferase with a follow-up necropsy and histological analysis of each mouse.

The IVIS confirmed that all tumors originated from the injected cells (Fig. 18). The signal of each tumor correlated very well with the measured size and position of the tumors (Fig.18). We concluded that there was no spontaneous tumor growth and that the injected cells survived throughout the teratoma assay since they could still metabolize the injected D-Luciferin.

The histological analysis revealed two types of tumors in both groups. The first type of tumors is the so called “florid” tumor. Florid tumors represent the fast-growing tumors in both groups and consisted of cells derived from the three germ layers, ectoderm (Fig. 19 Ai and Bi), mesoderm (Fig. 19 Aii and Bii) and endoderm (Fig. 19 Aiii and Biii). Therefore florid tumors can be classified as teratoma. Florid teratomas showed signs of vascularization (Fig. 19. A and B). The second type of tumor is the so called “atrophic” tumor. Atrophic tumors represent the slow or not growing tumors in both groups. Atrophic tumors were only found in

the lowest cell dosage of injected WT cells. The atrophic tumors consisted of cells derived from the three germ layers, ectoderm (Fig. 20 Ci and Di), mesoderm (Fig. 20 Cii and Dii) and endoderm (Fig. 20 Ciii and Diii). Therefore atrophic tumors can be classified as teratomas as well. Atrophic teratomas showed signs of degenerated nuclei and dead cells (Fig. 20 C and D). Furthermore they showed no signs of vascularization (Fig. 20 C and D). Both tumor types showed differences in the intensity of H&E staining between both groups. WT tumors tend to be paler than GoST cell tumors (Fig. 19 A and B, Fig 20 C and D). The H&E staining revealed that WT and GoST cell teratomas showed no sign of invasion or damaging of the surrounding tissue (Fig. 19 and 20).

The results of the teratoma assay showed that GoST induction does not prevent teratoma formation. However the tumor growth of GoST cells was reduced and the mice remained on average longer in the study compared to WT cells. We therefore concluded that the GoST induction effects the tumor growth potential. This effect is ambiguous though. As we observed downregulation of core pluripotency markers in GoST cells (D7), it is also possible, that the reduced tumor growth was caused by reduced pluripotency due to the removal of LIF during the one week of GoST induction.

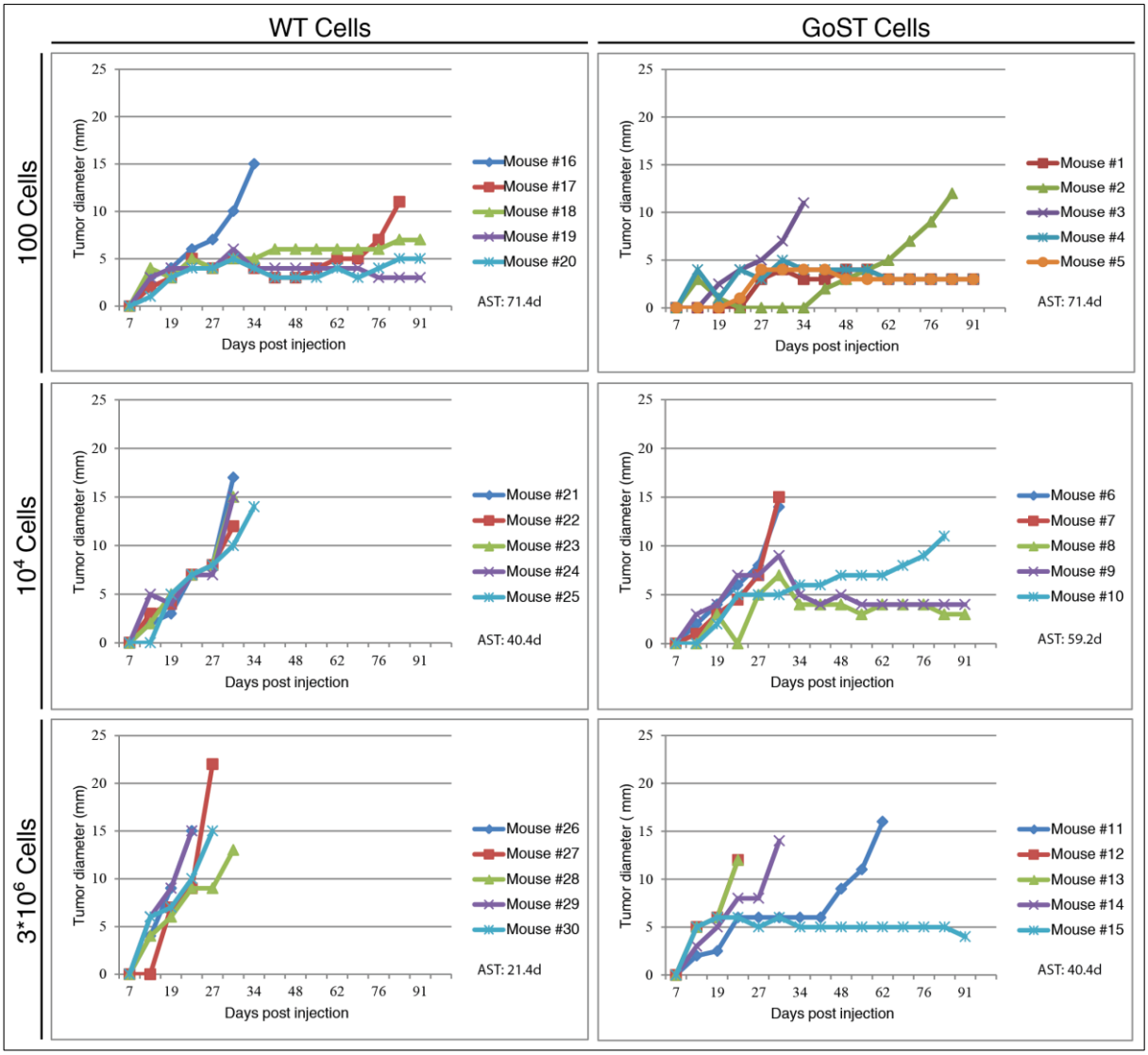


Fig. 17

Diameters of the tumors were measured manually using a sliding caliper once a week. The total study lasted 13 weeks (91 days). Each group consisted of 5 mice. End-point for the mice were tumor diameters >15 mm or a score >12 according to the score sheet (S5). AST = Average survival time.

Throughout the teratoma formation study differences in growth behavior between injected WT and GoST cells appeared. For the lowest cell dosage of 100 injected cells the growth curves look quite similar between WT and GoST cells with two mice reaching the end point of the animal experiment in each group at the same time (day 34 and day 79) and three mice staying in the experiment the full 13 weeks (91 days) with a steady tumor diameter around 3-6mm. The AST are equal in this cell dosage (71.4d). The curves are meandering around this value in the GoST cell group, but not in the WT group. In the 10^4 cell dosage differences between the two groups appeared. In the WT cells all mice reached the end point around the same time (day 30-34). In GoST cells only two mice reached the end point at day 30, but the remaining mice stayed longer in the experiment. It was observed that the diameter of the tumor of mouse #9 and #8 decreased after a peak around day 30. Only one mouse in the GoST cell group reached the end point after day 30 around day 79. Most of the GoST cell-derived

tumors did grow slowly. Measureable tumor growth e.g. in mouse #2 started at day 34 and had a slow growing curve, while in the WT cells tumors grew rapidly and the last mouse was sacrificed at day 34. The remaining mice in the GoST cell group had a similar tumor diameter of approximately 3mm, which did not increase over time. The AST was higher in the GoST cell group with 59.2 days compared to 40.4 days in the WT cell group. The highest injected cell dose of 3 million cells showed a similar result as the 10^4 cell dose in the WT group. The end point for 4 mice in the WT group was reached at around day 27 and the last mouse was sacrificed at day 30. In the GoST cell group, three mice reached the end point around day 27. One mouse reached the end point at day 62 and the last mouse stayed in the experiment for the full duration of 13 weeks. For mouse #11 the curve indicates a rapid growth of the tumor just starting at day 41 after staying at approximately 5 mm beforehand.

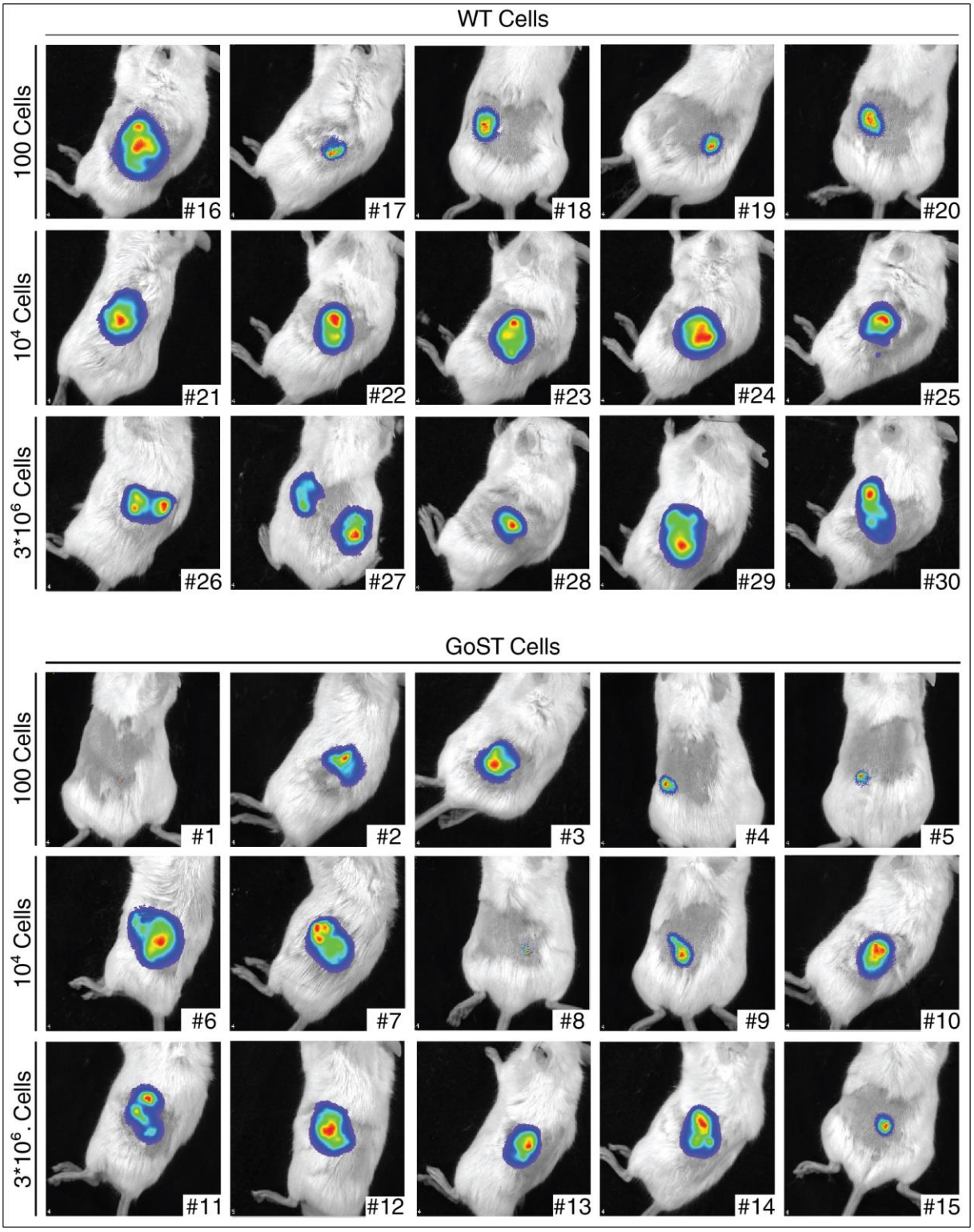


Fig. 18

IVIS images of mice 5-10 minutes after injection with D-Luciferin. The Signal is based on emitted photons and displayed by a color gradient from red (= high signal) to blue (=low signal).

The images show that every tumor originated from the luciferase positive cells that were injected. Size and region of the tumors correlate well with the measured diameter (Fig. 17) and the injection site. Although the

signal is very weak in mouse #1 and #8 there is still some signal detectable. Most of the mice show one hot spot with a high signal in the center and fading signal towards the periphery. Some mice like #11, #14 and #16 show several high signals in one hot spot with fading signals from the center to the outside. Mouse #26 and #27 show two separate hot spots which show one high signal in the center each with fading signal towards the periphery. The signals did not extend over the expected tumor size in WT and in GoST cell-derived tumors.

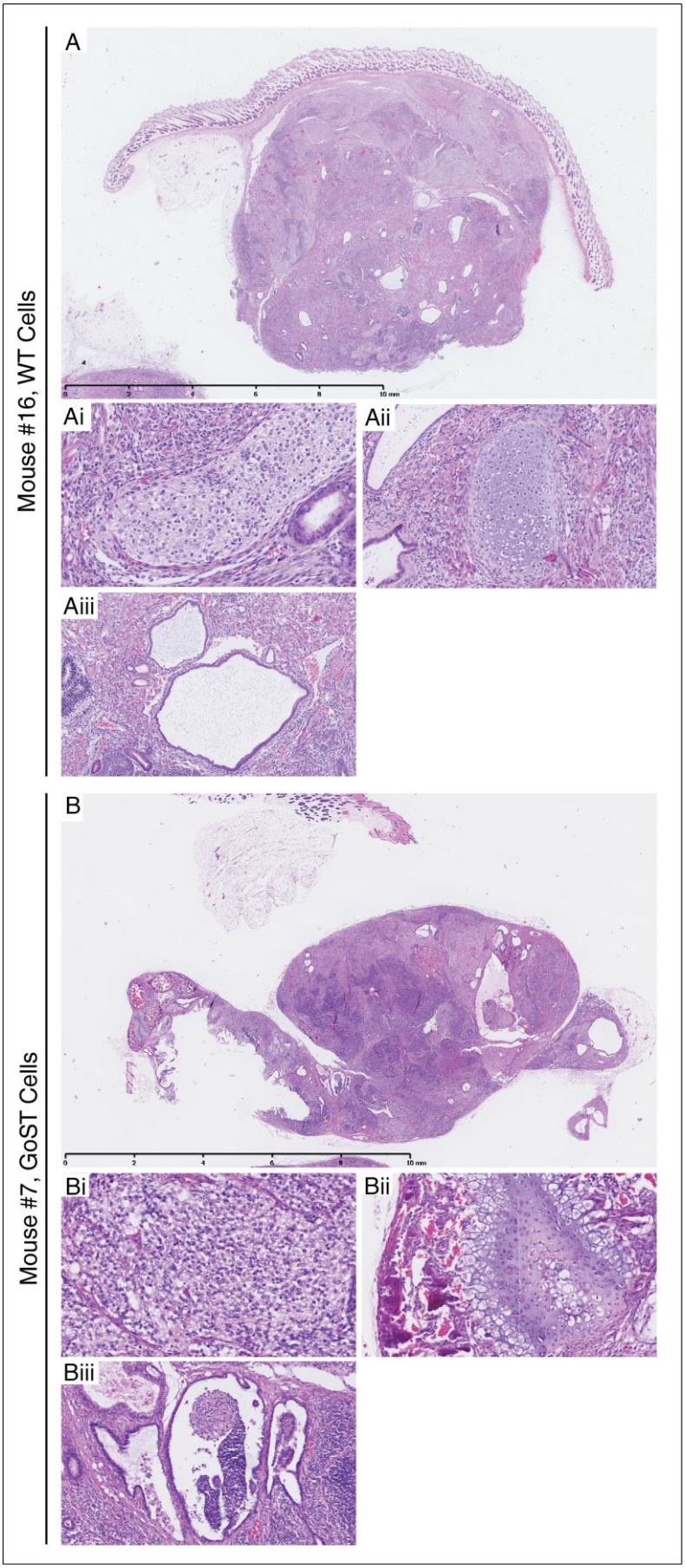

Mouse #16, WT Cells
A
Ai
Aii
Aiii
Mouse #7, GoST Cells
B
Bi
Bii
Biii

Fig. 19

H&E staining of paraffin embedded tumor sections. Images were taken from florid tumors representatively for other tumors of the same quality and morphology. Scale bar = 10mm

Fig. 19A panel represents the overview of a florid WT cell-tumor. The tumor only grew under the skin and did not infiltrate the surrounding tissue. Different parts of the tumor show variable intensities of staining. Image Ai shows an area of neuroectodermal tissue, which shows signs of primitive neurons and glia cells. Image Aii shows a piece of cartilage, which represents differentiated mesodermal cells. In Aiii dilated glandular tissue with cells inside and ciliated epithelium is visible. Image B represents the florid GoST cell tumors. Fig. 19B represents a florid tumor derived from GoST cells. The tumor did not infiltrate the surrounding tissue and was separated from the skin, which is visible at the edge of the image. The staining is more intense as in the WT tumor. In Bi an area of primitive neuroectodermal tissue is visible. Bii shows pieces of cartilage and pieces of bone inside the tumor, which both derived from the mesoderm. The endodermal offspring is shown in Biii with dilated glandular structures that contain clumps, which represent in this case neuroectodermal cells, and is equipped with ciliated epithelium on the inner cellular layer. The images show no difference in the constituent parts of the tumors that grew over 15 mm in diameter. Since the tumors did include tissue from all three germ-layers (ectoderm, mesoderm and endoderm) all florid and atrophic tumors classify as teratoma.

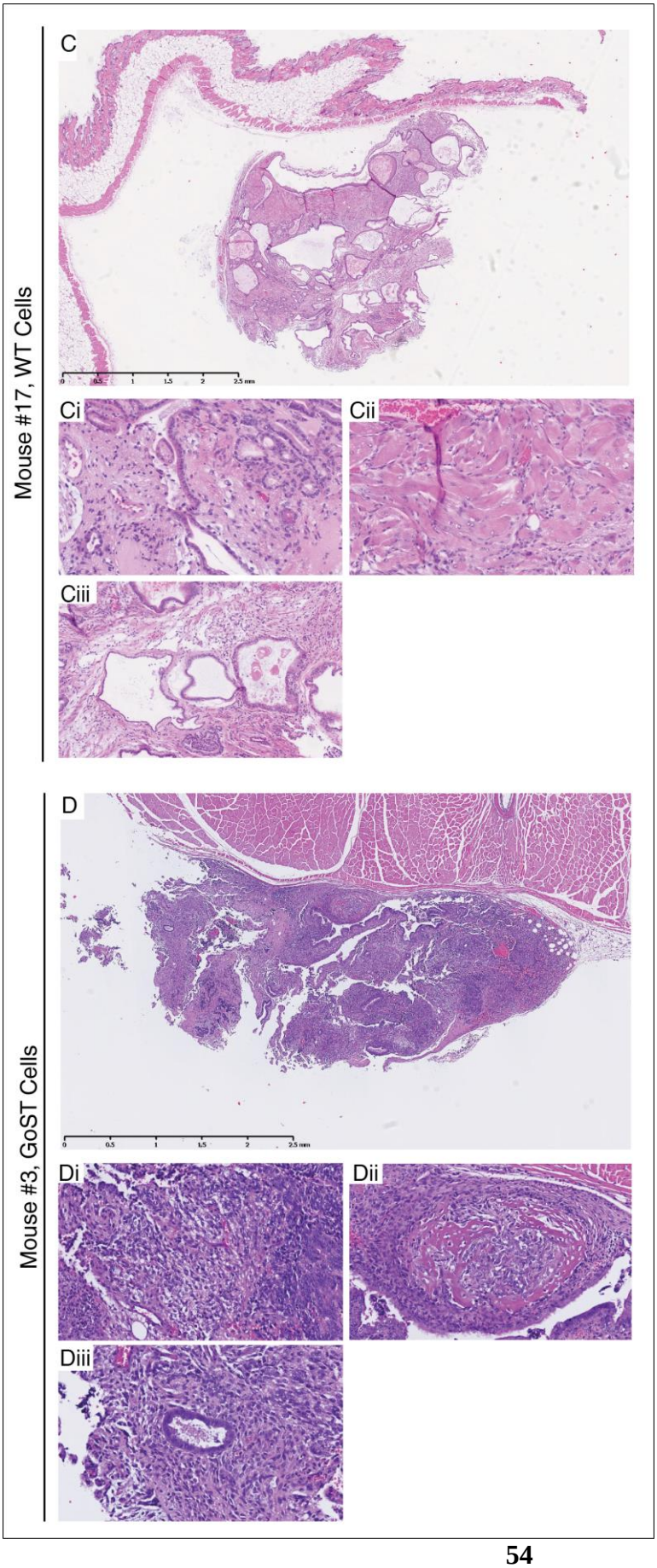

Mouse #17, WT Cells
C
Ci
Cii
Ciii
Mouse #3, GoST Cells
D
Di
Dii
Diii

Fig. 20

H&E staining of paraffin embedded atrophic tumor sections. Images were taken from atrophic tumors representatively for other atrophic tumors with the same quality and morphology. Scale bar = 2.5mm

Panel C represents the overview of an atrophic WT tumor. The tumor did not infiltrate its surrounding tissue and grew in the subcutaneous space. The staining shows similar intensity to the skin. In Ci nervous tissue, which represents the ectoderm, crossed by a glandular structure is shown. The cells lie in a high amount of matrix, which fills the space between the nuclei. In Cii skeletal muscle cells are visible, which originated from the mesoderm. Ciii shows a part of the tumor with several highly dilated glandular structures equipped with ciliated epithelium, which includes cells and fluids. Panel D shows the overview of a GoST cell-derived tumor. Intensity of the staining is higher than the surrounding tissue. The tumor is attached to the external layer of the abdominal muscle, but did not infiltrate the surrounding tissue. Image Di shows primitive neuroectodermal tissue. Dii shows a piece of cartilage, derived of mesodermal stem cells. The endodermal derivative is represented by a small glandular structure that lies in primitive neuroectodermal tissue. This gland is equipped with ciliated epithelium and contains cells and fluid that stained with H&E. Since both the WT- and GoST-derived tumors did contain cells from all three germ layers, these tumors can be classified as teratoma, similar to the florid tumors. The most significant differences between atrophic and florid tumors are the atrophic features and the lack of vascularization in atrophic tumors.

4.5 Injected cells showed signs of cellular migration

Since the teratoma assay showed promising results concerning the growth of tumors derived from GoST cells, we wanted to investigate a possible migration of stem cells from the injection site into the body of the mice. Since the injected cells were transgenic for β-galactosidase and luciferase, we isolated DNA from organs with subsequent PCR for luciferase to detect migrated transgenic cells. We have chosen typical bottlenecks in the body for migrating cells like liver, spleen and lung. For negative control of the transgenes we isolated DNA from earpunches of each mouse prior to injection. For detection of the injected cells we have chosen luciferase, since it was highly expressed in the injected cells (Fig. 21). As marker for DNA we have chosen Xist, an X-Chromosome silencing gene that is expressed in every cell independent of gender. This control was necessary since we expected no signal for luciferase in the organs. The initial plan to use a Y-Chromosome-specific gene as a second detection marker for the injected cells failed, since the injected cell did not have a Y-Chromosome (Fig.8).

In a preliminary experiment we analyzed the detectability of luciferin and Xist in earpunches and miPS clone 5 cells. The results showed that earpunches were negative for luciferase sequence in their genome while in miPS clone 5 cells were positive (Fig. 21).

We then investigated the DNA from organs and tumors, after the mice were sacrificed. The organs and tumor parts were pooled for each organ. The PCR results showed that Xist is detectable in all samples. Luciferase was not detected in spleen and liver samples (Fig. 22). Lung samples did show low amounts of signal for luciferase (Fig. 22). The tumors had the highest signal for luciferase which matches the previous observations of the miPS clone 5 cells (Fig. 5, 6, 7 and 21).

The necropsy of the mice could not find macroscopic evidence of migrated cells (Fig. 23). The organs appeared in physiological shape and color and no signs of metastasis of the teratoma were found (Fig. 22). The only difference appeared in the color of the teratoma during necropsy. WT teratoma did appear darker in color in both florid and atrophic teratoma (Fig. 23). GoST cell-derived teratoma did look pale compared to WT-derived teratoma, especially in the atrophic tumors (Fig. 20). Nevertheless there were tumors derived from WT cells, which were pale, and GoST cell-derived teratoma, which were darker (data not shown). In mouse #2 the teratoma did penetrate the abdominal wall and reached into the abdominal cavity, but without infiltrating or damaging the surrounding organs (Fig. 22).

The search for migrating cells revealed a potential migration of cells into the lungs after the injection. The migrated cells however did not form metastasis in the lung.

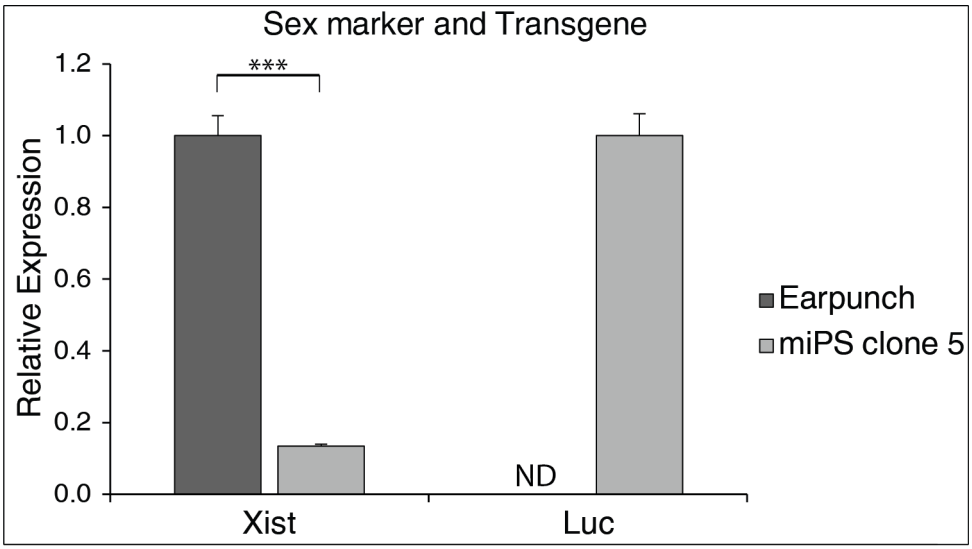


Fig. 21

RT-PCR of DNA from mouse tissue and miPS clone 5 cells. Data were generated from duplicates of three independent experiments. The error bars correspond to the S.E.M. One star represents $p<0.05$, two stars represent $p<0.01$, three stars represent $p<0.001$.

Prior to the injection of the cells, the mice were marked by earpunches. The earpunch-tissue taken from each mouse was collected and PCR of the X-Chromosome-specific gene Xist and the transgene luciferase was performed. Results show that the expression of Xist is significantly lower in miPS clone 5 cells compared to ear punch tissue, but still detectable. The transgene luciferase is not detectable in ear punches, but detectable in high amounts in the miPS clone 5 cells.

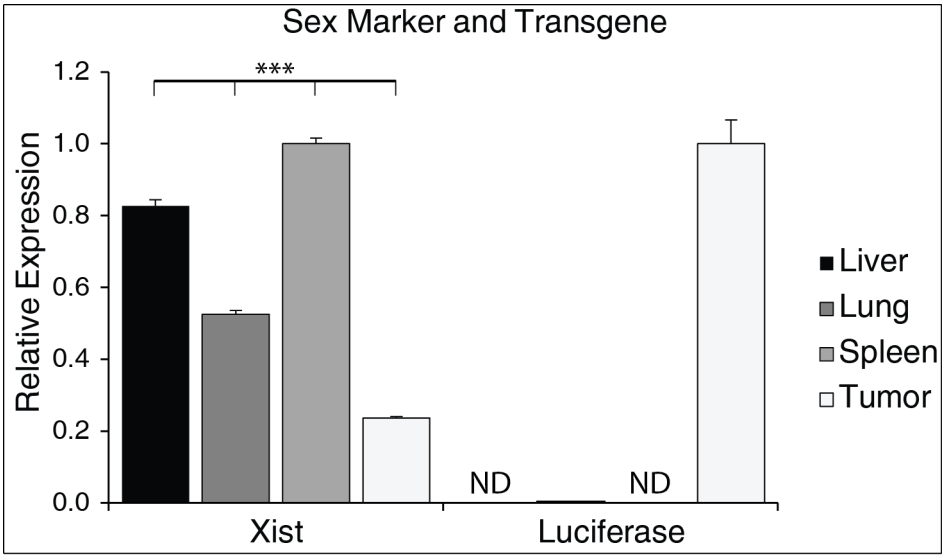


Fig. 22

RT-PCR of pooled DNA from extracted organs of the necropsy. Data were generated from duplicates of three independent experiments. The error bars correspond to the S.E.M. One star represents $p<0.05$, two stars represent $p< 0.01$, three stars represent $p<0.001$.

This analysis was conducted to analyze if luciferase positive cells migrated or metastasized after the injection. Since we could not detect the Y-chromosome-specific genes (Fig. 7), only luciferase or LacZ remained as tracers for these cells. We decided to use luciferase since it was sufficiently expressed in both miPS Luc/Lac and miPS clone 5 (Fig. 5). The X-chromosome-specific gene Xist is detectable in all organs and the difference in signal between all samples was highly significant. Luciferase was detected in tumor and lung samples.

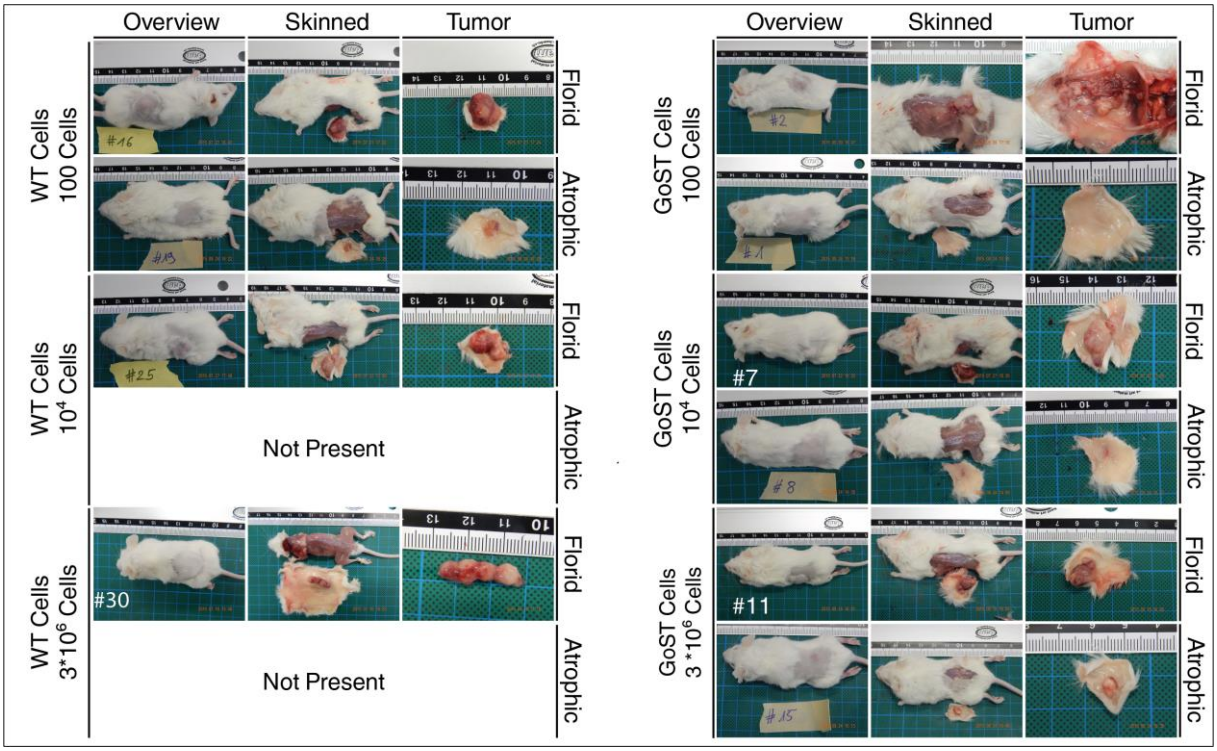


Fig. 23

Macroscopic pictures of injected mice after IVIS imaging and dissection. One square = 1x1cm, one dot ≈ 2mm

The necropsy after IVIS imaging gave more insights into the pathology of the tumors. In the overview images the tumors were easily visible from the external view for the tumors >15mm diameter. When skinned, most of the tumors did either attach to the skin or the abdominal wall, but could be separated easily and without alteration of the surrounding tissue. In mouse #2 the tumor did grow through the abdominal wall into the abdominal cavity, but did not infiltrate the surrounding tissue and organs. In other cases the GoST cell-derived florid teratomas stuck tightly to the skin and were not movable under the skin. Atrophic tumors were still visible with a small bump from the outside, but did not look as prominent as in the bigger tumors in both groups. WT and GoST cell-derived tumors did have a similar macroscopic morphology. They were of dark color and had a thin capsule that connected them to the skin or the abdominal wall. The GoST cell-derived florid teratoma did have a pale look compared to WT teratoma. Atrophic tumors showed some differences in the appearance between the two groups of injected cells. In WT cells, atrophic tumors did have a pink color similar to the florid tumors, while the GoST cell tumors did look pale, almost like the skin in the background. Atrophic tumors did only appear in the lowest cell dose of 100 injected cells in WT treated cells. In GoST cells the atrophic tumors appeared in all three cell dosages. Visceral organs did not show any pathologic alterations. The previously measured tumor diameters (Fig. 17) matched with the observed tumor diameters during the dissection.

5. Discussion

In the first step of this study we attempted to adapt the GoST induction from mES cells to miPS cell culture conditions in 2iL and further characterize GoST cells. This experiment proved that miPS cells survive the GoST induction culture conditions deprived of βME as described in mES cells[1] (Fig. 9). If the GoST induction induced germ cell-like features in miPS cells as well, needed to be addressed in further experiments.

By extending the unstimulated time period of miPS-derived GoST cells to 20 days we gained greater insights on mRNA expression patterns during that time period. We thereby observed a new phenotype of cells in the GoST cell population after 16 days (Fig. 10). The second goal was to investigate the tumor growth potential of miPS cell-derived GoST cells *in vivo.* Our results showed that GoST cells contributed to teratoma formation, but showed reduced tumor growth compared to WT cells.

The analysis of the core and naïve pluripotency markers shows that mES and miPS cells express the essential pluripotency markers Oct4, Sox2 and Nanog at similar levels, which confirms the literature[11,13,25] (Fig. 1 and 2). The immunofluorescence confirmed the real-time RT-PCR findings with similar staining intensity in mES and miPS clone 5 cells (Fig. 4). mEF cells, which represent the negative control, did not express core nor naïve pluripotency markers, which was expected for a differentiated cell type. The only pluripotency factor expressed in mEF cells was Klf4. This can be explained by the function of Klf4. Klf4 stabilizes the differentiation potential in mEF cells[128] and is therefore expressed in these cells. However, the expression of Klf4 in mEF cells was still significantly lower than in the PS cells. Immunofluorescence showed heterogeneous staining of TRA98 in both mES and miPS clone 5 cells. This might be an effect of heterogeneous TRA98 expression in PS cells, which is at least partially dependent on their cell cycle (Fig. 4).

The results of Western blot analysis on the other hand showed significant discrepancies between mES and miPS clone 5 cells for the expression of Nanog, Oct4 and Sox2 (Fig. 3). These results differ from the previous findings in mRNA quantification and immunofluorescence. A possible explanation might be technical and analytical errors of the Western blots, since the signal seems to decay towards one side of the blot and within the bands (Fig. 3). Nevertheless, mEF cells showed signs of expression of pluripotency markers in the analysis of the western blots (Fig. 3). This signal is most likely background noise of the western blots detected by the ImageJ software, since it is clearly visible that no bands appear

in mEF cells (Fig. 3). The analysis of the integrated transgenes in the miPS, mES and in the isolated cell clones clearly showed the presence of luciferase and LacZ in the cells (Figs. 5, 6 and 7). The X-Gal-staining showed distinct differences between non-transgenic and transgenic miPS cells (Fig. 5). There are however significant differences in the detectable amount of transgenes of luciferase and LacZ in the mES clone 11, miPS Luc/Lac and clone 5 cell lines (Fig. 7). It is possible that the plasmids integrated into the genome or decayed over time during passaging in the cell culture. This effect can be caused by unspecific multiple integration of the plasmid into the genome and might be insertion side dependent e.g. when integrated into certain promoter regions or silent integration into introns, which would affect the signal of the transgenes. Epigenetic modulation represents another important regulatory mechanism of gene expression and might be another reason for the reduced expression of transgenes. This effect could be evaluated by sequencing of the genome.
But nevertheless the results showed clearly that miPS clone 5 is a pluripotent stem cell line that expresses reliably the introduced reporter transgenes. Therefore the reporter transgenes represent a reliable tool to detect the cells *in vitro* or *in vivo*.

The analysis of the X/Y-chromosome-specific genes revealed that in the isolated cell clones 11 and 5 the Y-Chromosome-specific genes Sry and Zfy were not detectable while the primers showed specificity and consistent signal in other cell lines as well as in tissue samples (Fig. 7). After investigating the chromosome morphology via FISH painting the source of the lost signal was the total loss of the Y-Chromosome (Fig. 8B). The Y-Chromosome signal in the FISH chromosome painting of the X-Chromosome is most likely a false-positive result (Fig. 8A). The Y-Chromosome consists of high density chromatin and therefore does not recombine with other chromsomes[129]. Loss or the partial deletion of the Y-Chromosome occurs not only as a natural event[130,131] but can happen during cancer development[132,133] as well as during culturing of stem cells and is reported in several stem cell lines used for scientific purposes[113]. The differences in signal for the X-Chromosome-specific gene Xist might be due to technical errors and handling of the isolated DNA like different amounts of pipetted DNA. The elongation of the X-Chromosome can also be taken in consideration for the disparity of signal. This observation might explain the observed differences in transgene expression, since it is possible that not only the sex chromosomes show alterations but the autosomal chromosomes as well.

Closer examination during GoST induction confirmed the observation that the chemical treatment protects the cells from death when deprived from 2iL medium compared to the WT treated cells[1,73]. This is mainly ascribed by the attributes of the used chemicals, in particular tBHQ which prevents oxidative stress through Nrf2 signaling and thereby protecting the cells [76]. The possibility that other supplements made the survival possible, like DMSO or bare BM, can be ruled out since the WT cells did not survive culture in BM + DMSO (Fig. 9). Thus the GoST induction is the key factor for the survival of the cells. Usually the ingredient β-mercaptoethanol is essential for survival of the cells, since it raises the intracellular reduced form of GSH and thereby protects cells from toxic metabolites and oxidative stress and is a standard component of stem cell culture methods[134-136].

The mRNA expression profiles during the GoST induction period and the following unstimulated time period showed interesting results (Figs. 11-14). The core pluripotency markers decreased in both WT and GoST cells except for *Klf4*. Expression of core pluripotency markers of GoST cells were downregulated at D0 compared to WT cells. The upregulation of the transcriptional factor *Klf4* can be caused by differentiation processes since *Klf4*, like all Krüppel-like factors, is a potent regulator of differentiation[137]. Since WT cells show downregulation and GoST cells massive upregulation of *Klf4* over the whole examined time points, it indicates a decisive change in expression for GoST cells. Interestingly, WT cells showed downregulation of *Klf4* but still expressed germ cell markers like GoST cells. Most of the naïve pluripotency markers decrease as well in WT and GoST cells. Exceptions are *Piwil2* and *Rex1* in both groups. The increase and consistent expression of *Rex1* throughout the series in both groups represents a remarkable result, since it is reported that Nanog, Sox2 and Oct4 bind to the promotor region of Rex1[15,138] and downregulation of those factors should result in downregulation of Rex1[139]. This could mean other factors or effects independent from these pluripotency factors mediate the maintained expression of Rex1. The only differentiated tissue with constant Rex1 expression, besides stem cells, are testicles[140]. Otherwise a maintained Rex1 expression would indicate an undifferentiated state of the cells which can be excluded, since most of the pluripotency markers are downregulated, as mentioned above. *Piwil2* expression is linked to cell integrity and protection of DNA[141] especially in cancer[142,143]. This could indicate reorganization processes in the genome during the unstimulated time period in both WT and GoST cells. The upregulation showed the same trend in both cell types which hardens the suspicion that this effect results of the exclusion of

either the chemicals of the GoST induction or the 2i+Lif growth medium in WT cells. This pattern does not overlap with the *Rex1* expression though. *Piwil2* also was reportedly expressed in bovine testis, together with *Ddx4*, a germ cell marker[144].
As a conclusion of the analyzed pluripotency markers both WT and GoST cells have no longer pluripotent properties and must have differentiated. The *Rex1* and *Piwil2* expression indicates a germ cell-like state in both groups.

The analysis of germ cell-specific markers showed decreasing expression rates of *Dazl* and *Nanos2*. Their peak expression lies between 4-12 days of unstimulated culturing (Fig. 13). *Dazl* is a crucial gene linked to germ cell development[145] and *Nanos2* is an important factor for maintenance of the primitive state of spermatogonial stem cells[67]. This indicates a germ cell-like state during this time period for both groups. The expression peaks do show a correlation with the expression patterns of *Rex1*, *Piwil2* and *Klf4*, which showed their highest expression rates in this timeframe. This again can be related to a germ-cell like fate of the cells. *Klf4* stands out in this regard, since the expression does not decrease after 12 days of culturing in unstimulated environment in GoST cells, but in WT cells. *Rex1* and *Piwil2* expression increased over time and expression peaked around day 12 without stimulation. Interestingly the genes *Tdrd1*, *Tex101* and *Zbtb16* showed little to no expression in the PS cells, but increased massively throughout the 20 days. Fascinatingly Tex101, a highly specific marker for germ cells, is higher expressed in WT treated cells than in GoST cells. Peak expressions of *Dazl*, *Ddx4* and *Tdrd1* are higher in WT cells as well, but without significance. *Plk1s1* and *Zbtb16* expression rates stand out in this analysis, since the increase of expression in the GoST cells was significantly higher than in WT cells. This can be a result of higher activity of mitosis in the GoST cells during the unstimulated time period. Plk1s1 is a stabilizing factor for chromosome division[146] and Zbtb16, also known as PLZF, codes for a regulatory protein that plays an important role in cancer formation in male patients[147,148]. These results confirm a tendency towards a germ cell-like fate, which is more developed in GoST cells as in WT treated cells. The explanation is most likely that GoST induction enhances the differentiation of the cells towards the germ cell line after release of inhibiting factors. An interesting result is the expression of germ cell markers in WT cells.
The germ cell-like fate of both groups is also shown in the analysis of spermatogonial stem cell markers (Fig. 14). GoST cells showed higher and earlier expression of the spermatogonial stem cell markers Cd53, Csf1r, Gfra1 and Ifi203. This can be a result of differentiation

towards spermatogonial stem cells, which is more prevalent in GoST cells. The only exception is Col1a2, which shows a higher peak in WT cells early on, decreased to low levels and increased again with higher expression levels compared to GoST cells. Col1a2 is related to collagen formation and connectivity between cells[149]. Since the variety of tissues that express collagen is very high, no precise estimation can be made if WT cells differentiated differently than GoST cells. It is imaginable that the WT cells differentiated into connective tissue with germ cell features. The higher expressed collagen may be an explanation for the thicker cell layers in WT cells during the unstimulated time period (Fig. 10).

After all the real-time RT-PCR results indicate a differentiation path of both groups from pluripotent stem cells into at least germ-like cells. These differentiation pathways seem to overlap, but with small differences in the end points of differentiation. Since the major difference between both cell types is the appearance of round cells in GoST cells, these differences can be explained by the influence, differentiation and proliferation of those cells caused by the previous GoST induction. An unexpected result was that germ cell and SSC marker were expressed in WT cells as well. We expected that the miPS clone 5 cells would not express any germ cell markers since they did not underwent the GoST induction. This expression pattern of the WT cells might be influenced by the added 2iL in the medium and the overall lower incubation time (20 days) compared to GoST cells (27 days). This would explain why both groups showed a lot of similar expression patterns around the same time points since culture conditions were the same on both groups. It is also possible that this effect is unique to iPS cells. Since iPS cells might have an epigenetic memory this could influence the differentiation pathway during the unstimulated culture period[32]. Therefore both groups show similar expression patterns of germ and SSC markers. This effect is more prevalent in GoST cells though.

In ongoing work, following up on this study, WT cells were cultured for 7 days longer so that they were cultured in the absence of 2iL for the same duration as GoST cells. This new work revealed that WT cells also differentiated into round cells starting at around D23-D24.

The images taken from the GoST series hardened the suspicion for a germ cell-like fate. Since the WT cells did not change their morphology other than proliferating into thicker layers of cells between the colonies, which goes along quiet well with the increased *Col1a2* expression, the GoST cells did proliferate into small round cells from day 16 on which populated the entire culture dish until day 20. The reported morphology of spermatogonial stem cells

showed small round cells[64,65] together with the shown *TRA98* and the increasing *Gfra1* expression intensifies the suggestion that these cells have spermatogonial stem cell features on a molecular level. The WT cells did show *TRA98* expression in the unstimulated time period, but not in the former colonies (Fig. 15). The *TRA98* signal in WT cells can be residues of PS cells in the culture dish since these cells are found in the center of colonies (Fig. 16). It is possible that the cells rounded up due to overcrowding in the culture dish, which would lead to expulsion of cells to the medium. The continued TRA98 expression and the germ cell and SSC marker expression is a striking observation, since it would be expected that these cells would differentiate into neuroectodermal cells[150]. This should have been effecting both groups, but GoST cells did not grew as thick as the WT cells during the unstimulated time period. The GoST induction might be an explanation for the appearance of the round cells but, as mentioned before, the differences in incubation time in both groups has to be taken in account. The finding that WT and GoST cells showed the same expression patterns with peaks at the same time points across the investigated markers is another interesting result. Since GoST cells had 7 days of GoST induction prior to the unstimulated time period, the expression patterns were expected to be shifted in WT cells. Therefore the release of 2iL and the culture in unstimulated conditions is the dominating factor for the expression patterns of the tested markers.

After injecting GoST cells directly at the end of the GoST induction, they still contributed to tumor growth. The overall tumor formation potential did not match with WT cells (Fig. 17) though. The decrease of pluripotency factors at D7 (Figs. 11 and 12) explains the reduced tumor growth, since tumor growth potential is linked to pluripotency[116,126]. It might be possible that the cells were weakened by the GoST induction and therefore could not proliferate *in vivo* like the WT cells, as displayed by the shape of the nuclei at D7 (Fig. 16). This is unlikely since the WT cells did show the same growth characteristics in the lowest injected cell dose and were injected without any treatment. The appearance of the round cells in GoST cells as well indicates that the GoST cells were vital and were able to proliferate *in vitro* (Fig. 10). Histological analysis showed no sign of vascularization of atrophic teratomas in both groups, which explains the inhibited or total lack of growth. Nourishment of the tumors can only be supplied via diffusion to a certain extent and tumor growth is therefore limited to a certain size. The reason why some tumors did develop vascular supply might lie in a different activation of vascular growth factor (VGF) by the cells or the surrounding tissue

which is crucial to tumor formation and development[151]. The heterogeneous cell population in the GoST cells with flat and high cells can be another reason for the differences in tumor growth. The flat cell might represent differentiated cells and the thick cell layers remaining PS cells (Fig. 9). This argument does not hold true for the WT cell though. Since the WT cells were derived from a single cell clone there can't be differentiated subpopulations, which would have been visible in the immunofluorescence of pluripotency marker (Fig. 4). If a subpopulation would be injected, the growth curves should still show similar slopes compared to WT cells. It is more likely that this effect is connected to the impaired pluripotency at this time point. Since the hosts were highly immunocompromised animals, a host rejection can be ruled out as well.

The histological analysis of the tumors did not reveal any differences between both groups, independent of GoST treated cells or WT cells, injected cell number and florid or atrophic growth. This can be ascribed to the reduced, but not eliminated pluripotency of GoST cells. Therefore the resulting tumors still classify as teratomas in both groups.

The IVIS imaging and necropsy with the following screening for metastasis showed a positive signal in the lungs. Teratomas are usually non-malignant tumors and thus do not form metastasis, especially when the tumor does not have a vascular connection to the host. Since migrating cells would be expected to settle in terminal vessel systems, the lung is a likely organ for the cells. The observed differences in color of the tumors during necropsy can either be explained by the degree of vascularization of the tumors which, as mentioned before, could have been affected by the pluripotency of the injected cells. Since blood was drawn from each mouse after IVIS imaging it might be possible that the color of the tumors was dependent on the degree of exsanguination. The differences in color of the H&E staining can be ascribed to the predominant cell type or the cell density that each tumor consisted of. Since GoST cells showed different expression patterns on the mRNA level compared to WT cells, they may have a tendency towards forming high-density tissue *in vivo*, which WT cells don't.

6. Conclusion

The goal of this thesis was the characterization of miPS-derived GoST cells and the investigation of their tumor growth potential *in vivo* compared to WT miPS cells. The injection of GoST cells on D7 may have shown reduced tumor growth but did not prevent teratoma formation. On the other hand GoST cells showed the promising tendency to have slow or no further growth *in vivo* at a certain size of the tumors, even at high cell doses.

Further analysis of the GoST cells showed loss of core and naïve pluripotency markers as well as the increase of certain germ- and SSC markers. This implicates that tumorigenicity should decrease in the process. The *in vitro* results also showed the possibility of a spermatogonial stem cell differentiation pathway of GoST cells when cultured for 20 days without stimulation after GoST induction. This hypothesis is substantiated by the morphology of the round cells and the higher expressed SSC markers. The question, if the derived round cells actually represent SSCs and if they can be used for regenerative medicine e.g. for reproductive medicine, has to be addressed in further experiments. The similar expression pattern in WT cells and the differences in incubation time between GoST and WT cells are another issue that has to be investigated in further experiments.

7. Outlook

Although this study added new insights to the GoST induction, the distinct molecular and cellular mechanisms behind the GoST induction are still not fully understood. The results of this study raised many questions about the effect of the GoST induction on miPS cells. Thus, further investigations have to be made. Especially for the purpose of using GoST cells in reproductive medicine for the restoration of fertility, the issue of chromosomal alterations in miPS clone 5 cells has to be addressed. Therefore a new cell clone has to be isolated, cultured and screened again for chromosomal deficiencies. A regular screening during the whole culturing and GoST induction processes might be necessary. Novel techniques like the CRISPR/Cas9 system enable the creation of transgenic cells in a more precise and safe way to ensure genetic stability in these cells during culture. After the new clone is isolated, another teratoma assay has to be performed to evaluate the tumorigenicity of these cells as well. In future experiments the injection time may be shifted to another time point other than D7, since our new results indicate a promising change in GoST cells at day 20 with the appearance of the round cells. Steps should be taken to find specific markers of the round cells to isolate and sort only round cells. It should then be possible to inject only round cells. To ensure that the appearance of these cells is not a one-time event, the experiment should be repeated to ensure that the round cells are not somehow an artifact or arbitrary reaction of the cells to the GoST induction. A follow-up teratoma formation study confirmed the appearance of round cells in miPS-derived GoST cells after approximately 16 days after release of 2iL. The teratoma assay was conducted with the floating fraction of the round cells and confirmed that the round cells do not contribute to teratoma formation, but survive *in vivo*. Nevertheless,

further investigation of this new cell type is necessary. To explore these round cells more closely they should be directly compared to SSC's taken from mice testes. Real-time RT-PCR of mRNA and immunofluorescence with TRA98 and Gfra1 might be a valuable tool for identification of the round cells. This would also improve future data since this study lacked a positive control for germ cell and SSC markers. A mass spectrum analysis compared to actual spermatogonial stem cells might also be beneficial for a broader comparison between actual germ cells and the GoST-derived round cells. Murine SSC's can be obtained e.g. by isolation from neonatal or premature mice. Another interesting experiment could be the analysis of the DNA methylation grade of GoST cells during GoST induction and the unstimulated time period, since the degree of methylation is an important factor for germ cell development and has yet not been addressed in this study.

The final step would then be the injection of the round cells into a mouse testis. If the round cells represent SSC they should be capable of differentiating into fertile sperm when brought to their putative physiological location. For this experiment a busulfan treatment of mice could be used to sterilize the male mice before injection. Another possibility could be the usage of genetically sterile mice, which lack germ cells in the testis. A prerequisite for such experiments would be an intact blood-testis barrier to prevent host rejection. Transplantation of germ cells into germ cell-depleted testis has been shown to be very effective[152] and would fit the purpose of treating chemotherapy induced infertility in men as well. Since this study only used murine stem cells so far and the ultimate goal for GoST cell applications lies in human reproduction medicine, the GoST induction must be adapted to human iPS cells as well. Recent discoveries that lead back human ES cells from their primed to a naïve pluripotency cellular state might be of interest when adapting GoST induction to hiPS cells[23].

8. References

1 Moshfegh, C., Aires, L., Kisielow, M. & Vogel, V. A gonogenic stimulated transition of mouse embryonic stem cells with enhanced control of diverse differentiation pathways. *Scientific reports* **6**, 25104, doi:10.1038/srep25104 (2016).

2 Lee, A. S., Tang, C., Rao, M. S., Weissman, I. L. & Wu, J. C. Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies. *Nature medicine* **19**, 998-1004, doi:10.1038/nm.3267 (2013).

3 Gardner, R. L. and Brook, A. B. The origin and efficient derivation of embryonic stem cells in the mouse. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 5709–5712 (1997).

4 Martin, G. R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 7634-7638 (1981).

5 Mascetti, V. L. & Pedersen, R. A. Human-Mouse Chimerism Validates Human Stem Cell Pluripotency. *Cell stem cell* **18**, 67-72, doi:10.1016/j.stem.2015.11.017 (2016).

6 Lim, C. Y., Solter, D., Knowles, B. B. & Damjanov, I. Development of Teratocarcinomas and Teratomas in Severely Immunodeficient NOD.Cg-Prkdc(scid) Il2rg(tm1Wjl)/Szj (NSG) Mice. *Stem cells and development* **24**, 1515-1520, doi:10.1089/scd.2015.0033 (2015).

7 Silvan, U. *et al.* Angiogenesis and vascular network of teratocarcinoma from embryonic stem cell transplant into seminiferous tubules. *Br J Cancer* **101**, 64-70, doi:10.1038/sj.bjc.6605125 (2009).

8 Knoepfler, P. S. Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. *Stem cells* **27**, 1050-1056, doi:10.1002/stem.37 (2009).

9 Wray, J., Kalkan, T. & Smith, A. G. The ground state of pluripotency. *Biochemical Society transactions* **38**, 1027-1032, doi:10.1042/BST0381027 (2010).

10 Pashaiasl, M. *et al.* Unravelling evolution of Nanog, the key transcription factor involved in self-renewal of undifferentiated embryonic stem cells, by pattern recognition in nucleotide and tandem repeats characteristics. *Gene*, doi:10.1016/j.gene.2015.12.023 (2015).

11 Masui, S. *et al.* Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nature cell biology* **9**, 625-635, doi:10.1038/ncb1589 (2007).

12 Hall, J. *et al.* Oct4 and LIF/Stat3 additively induce Kruppel factors to sustain embryonic stem cell self-renewal. *Cell stem cell* **5**, 597-609, doi:10.1016/j.stem.2009.11.003 (2009).

13 Mitsui, K. *et al.* The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**, 631-642, doi:Doi 10.1016/S0092-8674(03)00393-3 (2003).

14 Torres, J. & Watt, F. M. Nanog maintains pluripotency of mouse embryonic stem cells by inhibiting NFkappaB and cooperating with Stat3. *Nature cell biology* **10**, 194-201, doi:10.1038/ncb1680 (2008).

15 Shi, W. *et al.* Regulation of the Pluripotency Marker Rex-1 by Nanog and Sox2. *Journal of Biological Chemistry* **281**, 23319-23325, doi:10.1074/jbc.M601811200 (2006).

16 Chiou, S. H. *et al.* Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. *Clin Cancer Res* **14**, 4085-4095, doi:10.1158/1078-0432.CCR-07-4404 (2008).
17 Cartwright, P. *et al.* LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* **132**, 885-896, doi:10.1242/dev.01670 (2005).
18 Jiang, J. *et al.* A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nature cell biology* **10**, 353-360, doi:10.1038/ncb1698 (2008).
19 Wei, D., Kanai, M., Huang, S. & Xie, K. Emerging role of KLF4 in human gastrointestinal cancer. *Carcinogenesis* **27**, 23-31, doi:10.1093/carcin/bgi243 (2006).
20 Saha, M. N., Abdi, J., Yang, Y. & Chang, H. miRNA-29a as a tumor suppressor mediates PRIMA-1Met-induced anti-myeloma activity by targeting c-Myc. *Oncotarget*, doi:10.18632/oncotarget.6880 (2016).
21 De Los Angeles, A., Loh, Y. H., Tesar, P. J. & Daley, G. Q. Accessing naive human pluripotency. *Current opinion in genetics & development* **22**, 272-282, doi:10.1016/j.gde.2012.03.001 (2012).
22 Nichols, J. & Smith, A. Naive and primed pluripotent states. *Cell stem cell* **4**, 487-492, doi:10.1016/j.stem.2009.05.015 (2009).
23 Guo, G. *et al.* Naive Pluripotent Stem Cells Derived Directly from Isolated Cells of the Human Inner Cell Mass. *Stem cell reports*, doi:10.1016/j.stemcr.2016.02.005 (2016).
24 Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-872, doi:10.1016/j.cell.2007.11.019 (2007).
25 Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676, doi:10.1016/j.cell.2006.07.024 (2006).
26 E. V. Grigor'eva, A. I. Z., A. I. Shevchenko, S. M. Zakian. Induced Pluripotent Stem Cells of Microtus levis x Microtus arvalis Vole Hybrids: Conditions Necessary for Their Generation and Self-Renewal. *Acta Naturae* **7**, 56-69 (2015).
27 Reimer, A. *et al.* Scalable topographies to support proliferation and Oct4 expression by human induced pluripotent stem cells. *Scientific reports* **6**, 18948, doi:10.1038/srep18948 (2016).
28 Katayama, M. *et al.* Induced pluripotent stem cells with six reprogramming factors from Prairie Vole, which is an animal model for social behaviors. *Cell transplantation*, doi:10.3727/096368916X690502 (2016).
29 Hernandez, D. *et al.* Electrical Stimulation Promotes Cardiac Differentiation of Human Induced Pluripotent Stem Cells. *Stem cells international* **2016**, 1718041, doi:10.1155/2016/1718041 (2016).
30 Dirja, B. T. *et al.* Potential of laryngeal muscle regeneration using induced pluripotent stem cell-derived skeletal muscle cells. *Acta Otolaryngol*, 1-6, doi:10.3109/00016489.2015.1126351 (2016).
31 Wang, H. *et al.* 5-HT2 receptors mediate functional modulation of GABAa receptors and inhibitory synaptic transmissions in human iPS-derived neurons. *Scientific reports* **6**, 20033, doi:10.1038/srep20033 (2016).
32 Kim, K. *et al.* Epigenetic memory in induced pluripotent stem cells. *Nature* **467**, 285-290, doi:10.1038/nature09342 (2010).
33 Miura, K. *et al.* Variation in the safety of induced pluripotent stem cell lines. *Nature biotechnology* **27**, 743-745, doi:10.1038/nbt.1554 (2009).

34 Cinalli, R. M., Rangan, P. & Lehmann, R. Germ cells are forever. *Cell* **132**, 559-562, doi:10.1016/j.cell.2008.02.003 (2008).
35 Molyneaux, K. & Wylie, C. Primordial germ cell migration. *The International journal of developmental biology* **48**, 537-544, doi:10.1387/ijdb.041833km (2004).
36 Raz, E. Guidance of primordial germ cell migration. *Current opinion in cell biology* **16**, 169-173, doi:10.1016/j.ceb.2004.01.004 (2004).
37 Richardson, B. E. & Lehmann, R. Mechanisms guiding primordial germ cell migration: strategies from different organisms. *Nature reviews. Molecular cell biology* **11**, 37-49, doi:10.1038/nrm2815 (2010).
38 Yamaji, M. *et al.* Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nature genetics* **40**, 1016-1022, doi:10.1038/ng.186 (2008).
39 Molyneaux, K. A. The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development* **130**, 4279-4286, doi:10.1242/dev.00640 (2003).
40 Takayama, T. *et al.* Sexually dimorphic expression of the novel germ cell antigen TEX101 during mouse gonad development. *Biology of reproduction* **72**, 1315-1323, doi:10.1095/biolreprod.104.038810 (2005).
41 Kurita, A. *et al.* Identification, cloning, and initial characterization of a novel mouse testicular germ cell-specific antigen. *Biology of reproduction* **64**, 935-945, doi:10.1095/biolreprod64.3.935 (2001).
42 Ohmura, M. *et al.* Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. *Archives of Histology and Cytology* **67**, 285-296, doi:10.1679/aohc.67.285 (2004).
43 Suzuki, A. & Saga, Y. Nanos2 suppresses meiosis and promotes male germ cell differentiation. *Genes & development* **22**, 430-435, doi:10.1101/gad.1612708 (2008).
44 Suzuki, H., Tsuda, M., Kiso, M. & Saga, Y. Nanos3 maintains the germ cell lineage in the mouse by suppressing both Bax-dependent and -independent apoptotic pathways. *Developmental biology* **318**, 133-142, doi:10.1016/j.ydbio.2008.03.020 (2008).
45 Ruggiu, M. *et al.* The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. *Nature* **389**, 73-77, doi:10.1038/37987 (1997).
46 Saitou, M. Germ cell specification in mice. *Current opinion in genetics & development* **19**, 386-395, doi:10.1016/j.gde.2009.06.003 (2009).
47 Murakami, K. *et al.* NANOG alone induces germ cells in primed epiblast in vitro by activation of enhancers. *Nature* **529**, 403-407, doi:10.1038/nature16480 (2016).
48 Jin, H. *et al.* Molecular characterization of a germ-cell-specific antigen, TEX101, from mouse testis. *Zygote* **14**, 201-208, doi:10.1017/S0967199406003753 (2006).
49 Li, W. *et al.* Tex101 is essential for male fertility by affecting sperm migration into the oviduct in mice. *Journal of molecular cell biology* **5**, 345-347, doi:10.1093/jmcb/mjt031 (2013).
50 Ohinata, Y. *et al.* Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* **436**, 207-213, doi:10.1038/nature03813 (2005).
51 Turner, C. A., Mack, D. H. & Davis, M. M. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* **77**, 297-306, doi:10.1016/0092-8674(94)90321-2 (1994).
52 Zwaka, T. P. & Thomson, J. A. A germ cell origin of embryonic stem cells? *Development* **132**, 227-233, doi:10.1242/dev.01586 (2005).

53 Heaney, J. D. *et al.* Germ cell pluripotency, premature differentiation and susceptibility to testicular teratomas in mice. *Development* **139**, 1577-1586, doi:10.1242/dev.076851 (2012).
54 Ohno, R. *et al.* A replication-dependent passive mechanism modulates DNA demethylation in mouse primordial germ cells. *Development* **140**, 2892-2903, doi:10.1242/dev.093229 (2013).
55 Hajkova, P. *et al.* Epigenetic reprogramming in mouse primordial germ cells. *Mechanisms of development* **117**, 15-23, doi:10.1016/s0925-4773(02)00181-8 (2002).
56 Seki, Y. *et al.* Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice. *Developmental biology* **278**, 440-458, doi:10.1016/j.ydbio.2004.11.025 (2005).
57 Seki, Y. *et al.* Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. *Development* **134**, 2627-2638, doi:10.1242/dev.005611 (2007).
58 De Rooij, D. G. & Russell, L. D. All you wanted to know about spermatogonia but were afraid to ask. *Journal of andrology* **21**, 776-798, doi:10.1002/j.1939-4640.2000.tb03408.x (2000).
59 Grisanti, L. *et al.* Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. *Stem cells* **27**, 3043-3052, doi:10.1002/stem.206 (2009).
60 Bellaiche, J. *et al.* Spermatogonial stem cell quest: nanos2, marker of a subpopulation of undifferentiated A spermatogonia in trout testis. *Biology of reproduction* **90**, 79, doi:10.1095/biolreprod.113.116392 (2014).
61 Sada, A., Suzuki, A., Suzuki, H. & Saga, Y. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. *Science* **325**, 1394-1398, doi:10.1126/science.1172645 (2009).
62 Costoya, J. A. *et al.* Essential role of Plzf in maintenance of spermatogonial stem cells. *Nature genetics* **36**, 653-659, doi:10.1038/ng1367 (2004).
63 Buageaw, A. *et al.* GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. *Biology of reproduction* **73**, 1011-1016, doi:10.1095/biolreprod.105.043810 (2005).
64 Cai, H. *et al.* Enrichment and culture of spermatogonia from cryopreserved adult bovine testis tissue. *Anim Reprod Sci*, doi:10.1016/j.anireprosci.2016.01.009 (2016).
65 Tayebeh Rastegar Ph.D., M. H. R. P. D., Soraya Parvari Ph.D., Maryam Baazm Ph.D. The interaction between Sertoli cells and luekemia inhibitory factor on the propagation and differentiation of spermatogonial stem cells in vitro. *Iran J Reproductive Medicine* **13**, 679-686 (2015).
66 Saga, Y. Function of Nanos2 in the male germ cell lineage in mice. *Cell Mol Life Sci* **67**, 3815-3822, doi:10.1007/s00018-010-0456-x (2010).
67 Zhou, Z. *et al.* RNA Binding Protein Nanos2 Organizes Post-transcriptional Buffering System to Retain Primitive State of Mouse Spermatogonial Stem Cells. *Developmental cell* **34**, 96-107, doi:10.1016/j.devcel.2015.05.014 (2015).
68 Sada, A., Hasegawa, K., Pin, P. H. & Saga, Y. NANOS2 acts downstream of glial cell line-derived neurotrophic factor signaling to suppress differentiation of spermatogonial stem cells. *Stem cells* **30**, 280-291, doi:10.1002/stem.790 (2012).
69 Buaas, F. W. *et al.* Plzf is required in adult male germ cells for stem cell self-renewal. *Nature genetics* **36**, 647-652, doi:10.1038/ng1366 (2004).

70 Pizzol, D., Bertoldo, A. & Foresta, C. Male infertility: biomolecular aspects. *Biomol Concepts* **5**, 449-456, doi:10.1515/bmc-2014-0031 (2014).
71 Orwig, K. E. & Schlatt, S. Cryopreservation and transplantation of spermatogonia and testicular tissue for preservation of male fertility. *J Natl Cancer Inst Monogr*, 51-56, doi:10.1093/jncimonographs/lgi029 (2005).
72 Medicine, T. P. C. o. t. A. S. f. R. Report on varicocele and infertility. *Fertility and sterility* **90**, S247-S249, doi:10.1016/j.fertnstert.2008.08.050 (2008).
73 Moshfegh, C. The use of gonogenic mechanisms for the control of embryonic stem cell differentiation and insights into the regulation of mesenchymal stem cell differentiation by extracellular matrix. *Diss. ETH Zurich* **21977**, doi:10.3929/ethz-a-010279075 (2014).
74 Saitou, M., Kagiwada, S. & Kurimoto, K. Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. *Development* **139**, 15-31, doi:10.1242/dev.050849 (2012).
75 Duan, X. *et al.* Antioxidant tert-butylhydroquinone ameliorates arsenic-induced intracellular damages and apoptosis through induction of Nrf2-dependent antioxidant responses as well as stabilization of anti-apoptotic factor Bcl-2 in human keratinocytes. *Free radical biology & medicine*, doi:10.1016/j.freeradbiomed.2016.02.009 (2016).
76 Eftekharzadeh, B., Maghsoudi, N. & Khodagholi, F. Stabilization of transcription factor Nrf2 by tBHQ prevents oxidative stress-induced amyloid beta formation in NT2N neurons. *Biochimie* **92**, 245-253, doi:10.1016/j.biochi.2009.12.001 (2010).
77 Lee, J. M., Moehlenkamp, J. D., Hanson, J. M. & Johnson, J. A. Nrf2-dependent activation of the antioxidant responsive element by tert-butylhydroquinone is independent of oxidative stress in IMR-32 human neuroblastoma cells. *Biochemical and biophysical research communications* **280**, 286-292, doi:10.1006/bbrc.2000.4106 (2001).
78 Khalfallah, O., Rouleau, M., Barbry, P., Bardoni, B. & Lalli, E. Dax-1 knockdown in mouse embryonic stem cells induces loss of pluripotency and multilineage differentiation. *Stem cells* **27**, 1529-1537, doi:10.1002/stem.78 (2009).
79 Schuldiner, M., Itskovitz-Eldor, J. & Benvenisty, N. Selective ablation of human embryonic stem cells expressing a "suicide" gene. *Stem cells* **21**, 257-265, doi:10.1634/stemcells.21-3-257 (2003).
80 Bauer, G. *et al.* In vivo biosafety model to assess the risk of adverse events from retroviral and lentiviral vectors. *Molecular therapy : the journal of the American Society of Gene Therapy* **16**, 1308-1315, doi:10.1038/mt.2008.93 (2008).
81 Choo, A. B. *et al.* Selection against undifferentiated human embryonic stem cells by a cytotoxic antibody recognizing podocalyxin-like protein-1. *Stem cells* **26**, 1454-1463, doi:10.1634/stemcells.2007-0576 (2008).
82 Andrews, P. W. Human teratocarcinomas. *Biochimica et biophysica acta* **948**, 17-36, doi:10.1016/0304-419X(88)90003-0 (1988).
83 Evans, M. J. & Kaufman, M. H. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154-156, doi:10.1038/292154a0 (1981).
84 Meuwissen, H. J., Gatti, R. A., Terasaki, P. I., Hong, R. & Good, R. A. Treatment of lymphopenic hypogammaglobulinemia and bone-marrow aplasia by transplantation of allogeneic marrow. Crucial role of histocompatiility matching. *N Engl J Med* **281**, 691-697, doi:10.1056/NEJM196909252811302 (1969).

85 Park, B., Yoo, K. H. & Kim, C. Hematopoietic stem cell expansion and generation: the ways to make a breakthrough. *Blood Res* **50**, 194-203, doi:10.5045/br.2015.50.4.194 (2015).
86 Smith, D. K., He, M., Zhang, C. L. & Zheng, J. C. The therapeutic potential of cell identity reprogramming for the treatment of aging-related neurodegenerative disorders. *Prog Neurobiol*, doi:10.1016/j.pneurobio.2016.01.006 (2016).
87 Golas, M. M. & Sander, B. Use of human stem cells in Huntington disease modeling and translational research. *Experimental neurology*, doi:10.1016/j.expneurol.2016.01.021 (2016).
88 Naaldijk, Y. *et al.* Effect of systemic transplantation of bone marrow-derived mesenchymal stem cells on neuropathology markers in APP/PS1 Alzheimer mice. *Neuropathol Appl Neurobiol*, doi:10.1111/nan.12319 (2016).
89 Csobonyeiova, M., Polak, S. & Danisovic, L. Recent approaches and challenges in iPSCs: modeling and cell-based therapy of Alzheimer's disease. *Rev Neurosci*, doi:10.1515/revneuro-2015-0054 (2016).
90 Kumar, A. *et al.* Dopaminergic-primed fetal liver mesenchymal stromal-like cells can reverse parkinsonian symptoms in 6-hydroxydopamine-lesioned mice. *Cytotherapy* **18**, 307-319, doi:10.1016/j.jcyt.2015.11.007 (2016).
91 Pen, A. E. & Jensen, U. B. Current status of treating neurodegenerative disease with induced pluripotent stem cells. *Acta Neurol Scand*, doi:10.1111/ane.12545 (2016).
92 Bago, J. R. *et al.* Therapeutically engineered induced neural stem cells are tumour-homing and inhibit progression of glioblastoma. *Nature communications* **7**, 10593, doi:10.1038/ncomms10593 (2016).
93 Laflamme, M. A. & Murry, C. E. Heart regeneration. *Nature* **473**, 326-335, doi:10.1038/nature10147 (2011).
94 Zhang, J. *et al.* Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circulation research* **104**, e30-41, doi:10.1161/CIRCRESAHA.108.192237 (2009).
95 Schwartz, S. D. *et al.* Embryonic stem cell trials for macular degeneration: a preliminary report. *The Lancet* **379**, 713-720, doi:10.1016/s0140-6736(12)60028-2 (2012).
96 Li, Y. *et al.* Reprogramming of blood cells into induced pluripotent stem cells as a new cell source for cartilage repair. *Stem cell research & therapy* **7**, 31, doi:10.1186/s13287-016-0290-7 (2016).
97 Hansel, M. C. *et al.* The Use of Induced Pluripotent Stem Cells for the Study and Treatment of Liver Diseases. *Curr Protoc Toxicol* **67**, 14 13 11-14 13 27, doi:10.1002/0471140856.tx1413s67 (2016).
98 Zhou, Q. *et al.* Complete Meiosis from Embryonic Stem Cell-Derived Germ Cells In Vitro. *Cell stem cell*, doi:10.1016/j.stem.2016.01.017 (2016).
99 Galuppo, A. G. Spermatogonial stem cells as a therapeutic alternative for fertility preservation of prepubertal boys. *Einstein (Sao Paulo)* **13**, 637-639, doi:10.1590/S1679-45082015RB3456 (2015).
100 Duggal, G., Heindryckx, B., Deroo, T. & De Sutter, P. Use of pluripotent stem cells for reproductive medicine: are we there yet? *The Veterinary quarterly* **34**, 42-51, doi:10.1080/01652176.2014.891061 (2014).
101 Hayashi, Y., Saitou, M. & Yamanaka, S. Germline development from human pluripotent stem cells toward disease modeling of infertility. *Fertility and sterility* **97**, 1250-1259, doi:10.1016/j.fertnstert.2012.04.037 (2012).

102 Ruzicka, J., Kulijewicz-Nawrot, M., Rodrigez-Arellano, J. J., Jendelova, P. & Sykova, E. Mesenchymal Stem Cells Preserve Working Memory in the 3xTg-AD Mouse Model of Alzheimer's Disease. *International journal of molecular sciences* **17**, doi:10.3390/ijms17020152 (2016).
103 Sharma, A. & Scott, C. T. The ethics of publishing human germline research. *Nature biotechnology* **33**, 590-592, doi:10.1038/nbt.3252 (2015).
104 Shulman, H. M. *et al.* Chronic graft-versus-host syndrome in man. *The American Journal of Medicine* **69**, 204-217, doi:10.1016/0002-9343(80)90380-0 (1980).
105 Ahmed, R. P., Ashraf, M., Buccini, S., Shujia, J. & Haider, H. Cardiac tumorigenic potential of induced pluripotent stem cells in an immunocompetent host with myocardial infarction. *Regenerative medicine* **6**, 171-178, doi:10.2217/rme.10.103 (2011).
106 Ben-David, U. & Benvenisty, N. The tumorigenicity of human embryonic and induced pluripotent stem cells. *Nature reviews. Cancer* **11**, 268-277, doi:10.1038/nrc3034 (2011).
107 Kanemura, H. *et al.* Tumorigenicity studies of induced pluripotent stem cell (iPSC)-derived retinal pigment epithelium (RPE) for the treatment of age-related macular degeneration. *PloS one* **9**, e85336, doi:10.1371/journal.pone.0085336 (2014).
108 Kawamura, A. *et al.* Teratocarcinomas Arising from Allogeneic Induced Pluripotent Stem Cell-Derived Cardiac Tissue Constructs Provoked Host Immune Rejection in Mice. *Scientific reports* **6**, 19464, doi:10.1038/srep19464 (2016).
109 Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821, doi:10.1126/science.1225829 (2012).
110 Hsu, P. D., Lander, E. S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157**, 1262-1278, doi:10.1016/j.cell.2014.05.010 (2014).
111 Liang, P. *et al.* CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein & cell* **6**, 363-372, doi:10.1007/s13238-015-0153-5 (2015).
112 Silvan, U. *et al.* Embryonic stem cell transplantation into seminiferous tubules: a model for the study of invasive germ cell tumors of the testis. *Cell transplantation* **20**, 637-642, doi:10.3727/096368910X536581 (2011).
113 Ayako Sugawara, K. G., Yusuke Sotomaru, Toshio Sofuni, and Toshio Ito. Current Status of Chromosomal Abnormalities in Mouse Embryonic Stem Cell Lines Used in Japan. *Comparative Medicine* **56**, 31-34 (2006).
114 Soto, D. A. & Ross, P. J. Pluripotent stem cells and livestock genetic engineering. *Transgenic research*, doi:10.1007/s11248-016-9929-5 (2016).
115 Bulic-Jakus, F., Katusic Bojanac, A., Juric-Lekic, G., Vlahovic, M. & Sincic, N. Teratoma: from spontaneous tumors to the pluripotency/malignancy assay. *Wiley interdisciplinary reviews. Developmental biology*, doi:10.1002/wdev.219 (2015).
116 Wesselschmidt, R. L. The teratoma assay: an in vivo assessment of pluripotency. *Methods in molecular biology* **767**, 231-241, doi:10.1007/978-1-61779-201-4_17 (2011).
117 Damjanov, I. & Andrews, P. W. The terminology of teratocarcinomas and teratomas. *Nature biotechnology* **25**, 1212; discussion 1212, doi:10.1038/nbt1107-1212a (2007).
118 Wendy Y. Zhang, P. E. d. A. a. J. C. W. Teratoma formation: A tool for monitoring pluripotency in stem cell research. *Stemcellbook*, doi:doi/10.3824/stembook.1.53.1 (2012).
119 George J. Bosl, M. D. a. R. J. M., M.D. Testicular Germ-Cell Cancer. *The New England Journal of Medicine* **337**, 248-252 (1997).

120 Bustamante-Marin, X., Garness, J. A. & Capel, B. Testicular teratomas: an intersection of pluripotency, differentiation and cancer biology. *The International journal of developmental biology* **57**, 201-210, doi:10.1387/ijdb.130136bc (2013).
121 Asan, Z., Kaymaz, H. & Kilitci, A. Spinal intramedullary mature cystic teratoma in an adult. *Br J Neurosurg*, 1-3, doi:10.3109/02688697.2015.1135876 (2016).
122 Li, Y., Yang, B., Song, L. & Yan, D. Mature teratoma of the spinal cord in adults: An unusual case. *Oncol Lett* **6**, 942-946, doi:10.3892/ol.2013.1519 (2013).
123 Akakpo, P. K., Derkyi-Kwarteng, L., Quayson, S. E., Gyasi, R. K. & Anim, J. T. Ovarian Tumors in Children and Adolescents: A 10-Yr Histopathologic Review in Korle-Bu Teaching Hospital, Ghana. *Int J Gynecol Pathol*, doi:10.1097/PGP.0000000000000257 (2015).
124 Bentivegna, E. *et al.* Surgical Outcomes After Debulking Surgery for Intraabdominal Ovarian Growing Teratoma Syndrome: Analysis of 38 Cases. *Ann Surg Oncol* **22 Suppl 3**, 964-970, doi:10.1245/s10434-015-4608-y (2015).
125 Logothetis, C. J., Samuels, M. L., Trindade, A. & Johnson, D. E. The growing teratoma syndrome. *Cancer* **50**, 1629-1635, doi:10.1002/1097-0142(19821015)50:8<1629::aid-cncr2820500828>3.0.co;2-1 (1982).
126 Gropp, M. *et al.* Standardization of the teratoma assay for analysis of pluripotency of human ES cells and biosafety of their differentiated progeny. *PloS one* **7**, e45532, doi:10.1371/journal.pone.0045532 (2012).
127 Haraguchi, S., Matsubara, Y. & Hosoe, M. Chick embryos can form teratomas from microinjected mouse embryonic stem cells. *Development, growth & differentiation*, doi:10.1111/dgd.12260 (2015).
128 El-Karim, E. A., Hagos, E. G., Ghaleb, A. M., Yu, B. & Yang, V. W. Kruppel-like factor 4 regulates genetic stability in mouse embryonic fibroblasts. *Mol Cancer* **12**, 89, doi:10.1186/1476-4598-12-89 (2013).
129 Sun, S. & Heitman, J. Should Y stay or should Y go: The evolution of non-recombining sex chromosomes. *BioEssays : news and reviews in molecular, cellular and developmental biology* **34**, 938-942, doi:10.1002/bies.201200064 (2012).
130 Pierre, R. V. & Hoagland, H. C. Age-Associated Aneuploidy - Loss of Y-Chromosome from Human Bone-Marrow Cells with Aging. *Cancer* **30**, 889-&, doi:Doi 10.1002/1097-0142(197210)30:4<889::Aid-Cncr2820300405>3.0.Co;2-1 (1972).
131 Dewan, S., Puscheck, E. E., Coulam, C. B., Wilcox, A. J. & Jeyendran, R. S. Y-chromosome microdeletions and recurrent pregnancy loss. *Fertility and sterility* **85**, 441-445, doi:10.1016/j.fertnstert.2005.08.035 (2006).
132 Noveski, P. *et al.* Loss of Y Chromosome in Peripheral Blood of Colorectal and Prostate Cancer Patients. *PloS one* **11**, e0146264, doi:10.1371/journal.pone.0146264 (2016).
133 Wong, H. Y. *et al.* TMSB4Y is a candidate tumor suppressor on the Y chromosome and is deleted in male breast cancer. *Oncotarget*, doi:10.18632/oncotarget.6743 (2015).
134 Janjic, D. & Wollheim, C. B. Effect of 2-mercaptoethanol on glutathione levels, cystine uptake and insulin secretion in insulin-secreting cells. *European journal of biochemistry / FEBS* **210**, 297-304 (1992).
135 Masashi Takahashi, T. N., Seizo Hamano, Masashige Kuwayama, Naomichi Okamura and Akira Okano. Effect of Thiol Compounds on In Vitro Development and

Intracellular Glutathione Content of Bovine Embryos. *Biology of reproduction* **49**, 228-223, doi:10.1095/biolreprod49.2.228 (1993).

136 Broxmeyer, H.-D. C. a. H. E. SIRT1 Deficiency Downregulates PTEN=JNK=FOXO1 Pathway to Block ROS-Induced Apoptosis in Mouse Embryonic Stem Cells. *Stem cells and Developement* **00**, 1-9, doi:10.1089=scd.2010.0465 (2010).

137 Pearson, R., Fleetwood, J., Eaton, S., Crossley, M. & Bao, S. Kruppel-like transcription factors: a functional family. *The international journal of biochemistry & cell biology* **40**, 1996-2001, doi:10.1016/j.biocel.2007.07.018 (2008).

138 Etti ben-Shushan, J. R. T., Lorraine J. Gudas and Yehuddit Bergman. Rex-1, a Gene Encoding a Transcription Factor Expressed in the Early Embryo, Is Regulated via Oct-3/4 and Oct-6 Binding to an Octamer Site and a Novel Protein, Rox-1, Binding to an Adjacent Site. *Molecular and cellular biology* **18**, 1866-1878 (1998).

139 Wang, J. *et al.* A protein interaction network for pluripotency of embryonic stem cells. *Nature* **444**, 364-368, doi:10.1038/nature05284 (2006).

140 Rogers, M. B., Hosler, B. A. & Gudas, L. J. Specific expression of a retinoic acid-regulated, zinc-finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. *Development* **113**, 815-824 (1991).

141 Siomi, M. C., Sato, K., Pezic, D. & Aravin, A. A. PIWI-interacting small RNAs: the vanguard of genome defence. *Nature Reviews Molecular Cell Biology* **12**, 246-258, doi:10.1038/nrm3089 (2011).

142 Yang, Y., Zhang, X., Song, D. & Wei, J. Piwil2 modulates the invasion and metastasis of prostate cancer by regulating the expression of matrix metalloproteinase-9 and epithelial-mesenchymal transitions. *Oncol Lett* **10**, 1735-1740, doi:10.3892/ol.2015.3392 (2015).

143 Qu, X., Liu, J., Zhong, X., Li, X. & Zhang, Q. PIWIL2 promotes progression of non-small cell lung cancer by inducing CDK2 and Cyclin A expression. *J Transl Med* **13**, 301, doi:10.1186/s12967-015-0666-y (2015).

144 Roovers, Elke F. *et al.* Piwi Proteins and piRNAs in Mammalian Oocytes and Early Embryos. *Cell reports* **10**, 2069-2082, doi:10.1016/j.celrep.2015.02.062 (2015).

145 Fu, X. F. *et al.* DAZ Family Proteins, Key Players for Germ Cell Development. *Int J Biol Sci* **11**, 1226-1235, doi:10.7150/ijbs.11536 (2015).

146 Oshimori, N., Ohsugi, M. & Yamamoto, T. The Plk1 target Kizuna stabilizes mitotic centrosomes to ensure spindle bipolarity. *Nature cell biology* **8**, 1095-1101, doi:10.1038/ncb1474 (2006).

147 Hsieh, C. L. *et al.* PLZF, a tumor suppressor genetically lost in metastatic castration-resistant prostate cancer, is a mediator of resistance to androgen deprivation therapy. *Cancer research* **75**, 1944-1948, doi:10.1158/0008-5472.CAN-14-3602 (2015).

148 Xiao, G. Q. *et al.* Loss of PLZF expression in prostate cancer by immunohistochemistry correlates with tumor aggressiveness and metastasis. *PloS one* **10**, e0121318, doi:10.1371/journal.pone.0121318 (2015).

149 Wenstrup, R. J., Cohn, D. H., Cohen, T. & Byers, P. H. Arginine for Glycine Substitution in the Triple-Helical Domain of the Products of One Alpha-2(I) Collagen Allele (Col1a2) Produces the Osteogenesis Imperfecta Type-Iv Phenotype. *Journal of Biological Chemistry* **263**, 7734-7740 (1988).

150 Qi-Long Ying, J. N., Ian Chambers and Austin Smith. BMP Induction of Id Proteins Suppresses Differentiation and Sustains Embryonic Stem Cell Self-Renewal in Collaboration with STAT3. *Cell* **115**, 281-292 (2003).

151 Carmeliet, P. & Jain, R. K. Angiogenesis in cancer and other diseases. *Nature* **407**, 249-257, doi:10.1038/35025220 (2000).
152 Gutierrez, K. *et al.* Gonadotoxic effects of busulfan in two strains of mice. *Reprod Toxicol*, doi:10.1016/j.reprotox.2015.09.002 (2015).

9. Acknowledgements

This work was created under great help and support of many people.

First, I'd like to thank my lovely girlfriend Susi for her tremendous patience during the whole time that I spend in Zurich while you were in Germany and we only had the weekends for each other. You stayed strong after our beautiful son Mattis was born and kept me up to date the whole time. Without your support, I couldn't have taken this exciting opportunity. Thank you so much for letting me do the MD I wanted so much.

I'd also like to thank Viola Vogel and Ulrich Bleul for giving me the opportunity to do my MD in the beautiful city of Zurich at the amazing laboratory of applied mechanobiology at ETH Zurich together with the Clinic for Reproductive Medicine at the Vetsuisse-Faculty Zurich. You gave me support and input when I needed and always had so much understanding for my family situation. I hope that everyone involved in this project benefited from my work and that the insights and collaborations will last beyond this work.

Shout outs to Lina Aires who helped me with my Western blots. We had so many nice talks about science, life and babies. I wish you, Pablo and your little Ines all the best for the future!

I also like to thank the whole Vogel lab for making these 14 month such a pleasant time. I miss you all and hope that we will meet again soon!

Special thanks to my namesake Giovanni Pellegrini from the LAMP of Vetsuisse-Faculty University Zurich. It was nice working with you and you did an excellent work on our mice!

Also I'd like to thank Aldona Pieńskova-Schelling and Claude Schelling for their great help to solve our Y-Chromosome puzzle.

And last but not least I thank my supervisor Cameron Moshfegh. You are the most dedicated scientist I ever met and it was a pleasure for me to work with you. I learned a lot about science from you and you were the best supervisor a student could ask for. Thank you for all your patience and advices you gave me. And of course thank you for my amazing fare-well gift. I wish you all the best for your future career and keep on building pylons!

10. Curriculum Vitae

Vorname Name	Sebastian Giovanni Rambow
Geburtsdatum	19.01.1990
Geburtsort	Neustrelitz
Nationalität	Deutsch

Schulausbildung

06/1996 – 06/2000	Grundschule Bergfeld, Bergfeld, Deutschland
07/2000 – 06/2003	Heinrich Schliemann Gymnasium, Neustrelitz, Deutschland
07/2003 – 07/2009	Gymnasium Carolinum, Neustrelitz, Deutschland

Höchster Schulabschluss

10.07.2009	Abitur, Gymnasium Carolinum, Neustrelitz, Deutschland

Studium

10/2009-03/2015	Veterinärmedizin, LMU München, München, Deutschland

Abschlussprüfung vet. med.

24.03.2015	LMU München, München, Deutschland

Anfertigung der Dissertation

08/2015 – 05/2017	unter Leitung von Prof. Dr. med. vet. Ulrich Bleul, Dipl. ECBHM, Abteilung für Grosstierreproduktion, Klinik für Reproduktionsmedizin, Direktor: Prof. Dr. med. vet. Dipl. ECBHM Heiner Bollwein, Vetsuisse-Fakultät Universität Zürich

Alle fachrelevanten Anstellungen nach Abschluss des veterinärmedizinischen Studiums

06/2015 – heute	Assistenzarzt, Tierarztpraxis Dr. Johannes Hermann, Dachsbach, Deutschland

11. Supplements

S1

Lenti EF1-LacZ sequence

caggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataacc
ctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattccctttttgcggcattttgccttcc
tgtttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctcaa
cagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggtattatccc
gtattgacgccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcat
cttacggatggcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatc
ggaggaccgaaggagctaaccgctttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaag
ccataccaaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactcta
gcttcccggcaacaattaatagactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttatt
gctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttat
ctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgt
cagaccaagtttactcatatatactttagattgatttaaaacttcatttttaatttaaaaggatctaggtgaagatccttttgataatctcatgac
caaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatccttttttctgcgcgt
aatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaact
ggcttcagcagagcgcagataccaaatactgtccttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctaca
tacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccgg
ataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagataccta
cagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacagga
gagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgt
gatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttgctggccttttgctcaca
tgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacgaccgag
cgcagcgagtcagtgagcgaggaagcggaagagcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcattaatgcag
ctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggct
ttacactttatgcttccggctcgtatgttgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacgccaa
gcgcgcaattaaccctcactaaagggaacaaaagctggagctgcaagcttaatgtagtcttatgcaatactcttgtagtcttgcaacatgg
taacgatgagttagcaacatgccttacaaggagagaaaaagcaccgtgcatgccgattggtggaagtaaggtggtacgatcgtgcctt
attaggaaggcaacagacgggtctgacatggattggacgaaccactgaattgccgcattgcagagatattgtatttaagtgcctagctcg
atacaataaacgggtctctctggttagaccagatctgagcctgggagctctctggctaactagggaacccactgcttaagcctcaataaa

gcttgccttgagtgcttcaagtagtgtgtgcccgtctgttgtgtgactctggtaactagagatccctcagacccttttagtcagtgtggaaaa
tctctagcagtggcgcccgaacagggacctgaaagcgaaagggaaaccagagctctctcgacgcaggactcggcttgctgaagcgc
gcacggcaagaggcgaggggcggcgactggtgagtacgccaaaaattttgactagcggaggctagaaggagagagatgggtgcg
agagcgtcagtattaagcgggggagaattagatcgcgatgggaaaaaattcggttaaggccaggggaaagaaaaaatataaattaa
aacatatagtatgggcaagcagggagctagaacgattcgcagttaatcctggcctgttagaaacatcagaaggctgtagacaaatactg
ggacagctacaaccatcccttcagacaggatcagaagaacttagatcattatataatacagtagcaaccctctattgtgtgcatcaaagg
atagagataaaagacaccaaggaagctttagacaagatagaggaagagcaaaacaaaagtaagaccaccgcacagcaagcggccg
ctgatcttcagacctggaggaggagatatgagggacaattggagaagtgaattatataaatataaagtagtaaaaattgaaccattagga
gtagcacccaccaaggcaaagagaagagtggtgcagagagaaaaaagagcagtgggaataggagctttgttccttgggttcttggga
gcagcaggaagcactatgggcgcagcctcaatgacgctgacggtacaggccagacaattattgtctggtatagtgcagcagcagaac
aatttgctgagggctattgaggcgcaacagcatctgttgcaactcacagtctggggcatcaagcagctccaggcaagaatcctggctgt
ggaaagatacctaaaggatcaacagctcctggggatttggggttgctctggaaaactcatttgcaccactgctgtgccttggaatgctag
ttggagtaataaatctctggaacagattggaatcacacgacctggatggagtgggacagagaaattaacaattacacaagcttaatacac
tccttaattgaagaatcgcaaaaccagcaagaaaagaatgaacaagaattattggaattagataaatgggcaagtttgtggaattggttta
acataacaaattggctgtggtatataaaattattcataatgatagtaggaggcttggtaggtttaagaatagtttttgctgtactttctatagtg
aatagagttaggcagggatattcaccattatcgtttcagacccacctcccaaccccgaggggacccgacaggcccgaaggaatagaa
gaagaaggtggagagagagacagagacagatccattcgattagtgaacggatctcgacggtatcggttaacttttaaaagaaaaggg
gggattgggggtacagtgcaggggaaagaatagtagacataatagcaacagacatacaaactaaagaattacaaaaacaaattaca
aaaattcaaaattttccgatcacgagactagcctcgagagctagcggctccggtgcccgtcagtgggcagagcgcacatcgcccaca
gtccccgagaagttgggggggaggggtcggcaattgaaccggtgcctagagaaggtggcgcggggtaaactgggaaagtgatgtcg
tgtactggctccgccttttcccgagggtgggggagaaccgtatataagtgcagtagtcgccgtgaacgttcttttcgcaacgggtttgc
cgccagaacacaggtaagtgccgtgtgtggttcccgcgggcctggcctctttacgggttatggcccttgcgtgccttgaattacttccact
ggctgcagtacgtgattcttgatcccgagcttcgggttggaagtgggtgggagagttcgaggccttgcgcttaaggagccccttcgcct
cgtgcttgagttgaggcctggcctgggcgctggggccgccgcgtgcgaatctggtggcaccttcgcgcctgtctcgctgctttcgataa
gtctctagccatttaaaatttttgatgacctgctgcgacgctttttttctggcaagatagtcttgtaaatgcgggccaagatctgcacactggt
atttcggtttttggggccgcgggcggcgacggggcccgtgcgtcccagcgcacatgttcggcgaggcggggcctgcgagcgcggc
caccgagaatcggacgggggtagtctcaagctggccggcctgctctggtgcctggcctcgcgccgccgtgtatcgccccgccctgg
gcggcaaggctggcccggtcggcaccagttgcgtgagcggaaagatggccgcttcccggccctgctgcagggagctcaaaatgga
ggacgcggcgctcgggagagcgggcgggtgagtcacccacacaaaggaaaagggcctttccgtcctcagccgtcgcttcatgtgac
tccacggagtaccgggcgccgtccaggcacctcgattagttctcgagcttttggagtacgtcgtctttaggttgggggagggggttttat
gcgatggagtttccccacactgagtgggtggagactgaagttaggccagcttggcacttgatgtaattctccttggaatttgccctttttga
gtttggatcttggttcattctcaagcctcagacagtggttcaaagttttttttcttccatttcaggtgtcgtgagcgatcgccatggaccctgttg
tgctgcaaaggagagactgggagaaccctggagtgacccagctcaacagactggctgcccaccctcccttgcctcttggaggaactc
tgaggaagccaggacagacaggcccagccagcagctcaggtctctcaatggagagtggaggtttgcctggttccctgcccctgaagc

tgtgcctgagtcttggctggagtgtgacctcccagaggctgacactgttgtggtgcccagcaactggcagatgcatggctatgatgccc
ccatctacaccaatgtcacctaccccatcactgtgaaccccccttttgtgcccactgagaaccccactggctgctacagcctgaccttcaa
tgttgatgagagctggctgcaagaaggccagaccaggatcatctttgatggagtcaactctgccttccacctctggtgcaatggcaggt
gggttggctatggccaagacagcaggctgccctctgagtttgacctctctgccttcctcagagctggagagaacaggctggctgtcatg
gtgctcaggtggtctgatggcagctacctggaagaccaagacatgtggaggatgtctggcatcttcagggatgtgagcctgctgcaca
agcccaccacccagatttctgacttccatgttgccaccaggttcaatgatgacttcagcagagctgtgctggaggctgaggtgcagatgt
gtggagaactcagagactacctgagagtcacagtgagcctctggcaaggtgagacccaggtggcctctggcacagccccctttggag
gagagatcattgatgagagaggaggctatgctgacagagtcaccctgaggctcaatgtggagaaccccaagctgtggtctgctgagat
ccccaacctctacagggctgttgtggagctgcacactgctgatggcaccctgattgaagctgaagcctgtgatgttggattcagagaagt
caggattgagaatggcctgctgctgctcaatggcaagcctctgctcatcaggggagtcaacaggcatgagcaccaccctctgcatgga
caagtgatggatgaacagacaatggtgcaagatatcctgctaatgaagcagaacaacttcaatgctgtcaggtgctctcactaccccaa
ccaccctctctggtacaccctgtgtgacaggtatggcctgtatgttgttgatgaagccaacattgagacacatggcatggtgcccatgaa
caggctcacagatgaccccaggtggctgcctgccatgtctgagagagtgaccaggatggtgcagagagacaggaaccacccctctg
tgatcatctggtctctgggcaatgagtctggacatggagccaaccatgatgctctctacaggtggatcaagtctgttgaccccagcagac
ctgtgcagtatgaaggaggtggagcagacaccacagccacagacatcatctgccccatgtatgccagggttgatgaggaccagccct
tccctgctgtgcccaagtggagcatcaagaagtggctctctctgcctggagagaccagacctctgatcctgtgtgaatatgcacatgcaa
tgggcaactctctgggaggctttgccaagtactggcaagccttcagacagtaccccaggctgcaaggaggatttgtgtgggactgggt
ggaccaatctctcatcaagtatgatgagaatggcaacccctggtctgcctatggaggagactttggtgacacccccaatgacaggcagt
tctgcatgaatggcctggtctttgcagacaggacccctcaccctgccctcacagaggccaagcaccagcaacagttcttccagttcagg
ctgtctggacagaccattgaggtgacatctgagtacctcttcaggcactctgacaatgagctcctgcactggatggtggccctggatggc
aagcctctggcttctggtgaggtgcctctggatgtggcccctcaaggaaagcagctgattgaactgcctgagctgcctcagccagagtc
tgctggacaactgtggctaacagtgagggtggttcagcccaatgcaacagcttggtctgaggcaggccacatctctgcatggcagcag
tggaggctggctgagaacctctctgtgaccctgcctgctgcctctcatgccatccctcacctgacaacatctgaaatggacttctgcattg
agctgggcaacaagagatggcagttcaacaggcagtctggcttcctgtctcagatgtggattggagacaagaagcagctcctcacccc
tctcagggaccaattcaccagggctcctctggacaatgacattggagtgtctgaggccaccaggattgacccaaatgcttgggtggag
aggtggaaggctgctggacactaccaggctgaggctgccctgctccagtgcacagcagacaccctggctgatgctgttctgatcacca
cagcccatgcttggcagcaccaaggcaagaccctgttcatcagcagaaagacctacaggattgatggctctggacagatggcaatca
cagtggatgtggaggttgcctctgacacacctcaccctgcaaggattggcctgaactgtcaactggcacaggtggctgagagggtgaa
ctggctgggcttaggccctcaggagaactaccctgacaggctgacagctgcctgctttgacaggtgggacctgcctctgtctgacatgt
acacccctatgtgttcccttctgagaatggcctgaggtgtggcaccagggagctgaactatggtcctcaccagtggaggggagacttc
cagttcaacatctccaggtactctcagcaacagctcatggaaacctctcacaggcacctgctccatgcagaggagggaacctggctga
acattgatggcttccacatgggcattggaggagatgactcttggtctccttctgtgtctgctgagttccagttatctgctggcaggtaccact
atcagctggtgtggtgccagaagtaagtttaaacgcgtcgacaatcaacctctggattacaaaatttgtgaaagattgactggtattcttaa
ctatgttgctccttttacgctatgtggatacgctgctttaatgcctttgtatcatgctattgcttcccgtatggctttcattttctcctccttgtataa

atcctggttgctgtctctttatgaggagttgtggcccgttgtcaggcaacgtggcgtggtgtgcactgtgtttgctgacgcaacccccact
ggttggggcattgccaccacctgtcagctcctttccgggactttcgctttcccctccctattgccacggcggaactcatcgccgcctgcc
ttgcccgctgctggacaggggctcggctgttgggcactgacaattccgtggtgttgtcggggaagctgacgtcctttccatggctgctcg
cctgtgttgccacctggattctgcgcgggacgtccttctgctacgtcccttcggccctcaatccagcggaccttccttcccgcggcctgct
gccggctctgcggcctcttccgcgtcttcgccttcgccctcagacgagtcggatctccctttgggccgcctccccgcctggaattcgag
ctcggtacctttaagaccaatgacttacaaggcagctgtagatcttagccacttttaaaagaaaaggggggactggaagggctaattca
ctcccaacgaagacaagatctgctttttgcttgtactgggtctctctggttagaccagatctgagcctgggagctctctggctaactaggg
aacccactgcttaagcctcaataaagcttgccttgagtgcttcaagtagtgtgtgcccgtctgttgtgtgactctggtaactagagatccct
cagacccttttagtcagtgtggaaaatctctagcagtagtagttcatgtcatcttattattcagtatttataacttgcaaagaaatgaatatcag
agagtgagaggaacttgtttattgcagcttataatggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttcactgca
ttctagttgtggtttgtccaaactcatcaatgtatcttatcatgtctggctctagctatcccgcccctaactccgcccagttccgcccattctcc
gccccatggctgactaatttttttttatttatgcagaggccgaggccgcctcggcctctgagctattccagaagtagtgaggaggctttttg
gaggcctaggcttttgcgtcgagacgtacccaattcgccctatagtgagtcgtattacgcgcgctcactggccgtcgttttacaacgtcgt
gactgggaaaaccctggcgttacccaacttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgca
ccgatcgcccttcccaacagttgcgcagcctgaatggcgaatggcgcgacgcgccctgtagcggcgcattaagcgcggcgggtgtg
gtggttacgcgcagcgtgaccgctacacttgccagcgccctagcgcccgctcctttcgctttcttcccttcctttctcgccacgttcgccg
gctttccccgtcaagctctaaatcgggggctcccttagggttccgatttagtgctttacggcacctcgaccccaaaaaacttgattagggt
gatggttcacgtagtgggccatcgccctgatagacggtttttcgccctttgacgttggagtccacgttctttaatagtggactcttgttccaa
actggaacaacactcaaccctatctcggtctattcttttgatttataagggattttgccgatttcggcctattggttaaaaaatgagctgattta
acaaaaatttaacgcgaattttaacaaaatattaacgtttacaatttcc

S2

Lenti-CAG-puro-E2A-Luciferase sequence

caggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataacc
ctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattcccttttttgcggcattttgccttcc
tgtttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctcaa
cagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggtattatccc
gtattgacgccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcat
cttacggatggcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatc
ggaggaccgaaggagctaaccgcttttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaag
ccataccaaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactcta
gcttcccggcaacaattaatagactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttatt
gctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttat

ctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgt
cagaccaagtttactcatatatactttagattgatttaaaacttcatttttaatttaaaaggatctaggtgaagatccttttgataatctcatgac
caaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatccttttttctgcgcgt
aatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaact
ggcttcagcagagcgcagataccaaatactgtccttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctaca
tacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccgg
ataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagataccta
cagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacagga
gagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgt
gatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttgctggccttttgctcaca
tgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacgaccgag
cgcagcgagtcagtgagcgaggaagcggaagagcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcattaatgcag
ctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggct
ttacactttatgcttccggctcgtatgttgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacgccaa
gcgcgcaattaaccctcactaaagggaacaaaagctggagctgcaagcttaatgtagtcttatgcaatactcttgtagtcttgcaacatgg
taacgatgagttagcaacatgccttacaaggagagaaaaagcaccgtgcatgccgattggtggaagtaaggtggtacgatcgtgcctt
attaggaaggcaacagacgggtctgacatggattggacgaaccactgaattgccgcattgcagagatattgtatttaagtgcctagctcg
atacaataaacgggtctctctggttagaccagatctgagcctgggagctctctggctaactagggaacccactgcttaagcctcaataaa
gcttgccttgagtgcttcaagtagtgtgtgcccgtctgttgtgtgactctggtaactagagatccctcagacccttttagtcagtgtggaaaa
tctctagcagtggcgcccgaacagggacctgaaagcgaaagggaaaccagagctctctcgacgcaggactcggcttgctgaagcgc
gcacggcaagaggcgaggggcggcgactggtgagtacgccaaaaattttgactagcggaggctagaaggagagagatgggtgcg
agagcgtcagtattaagcgggggagaattagatcgcgatgggaaaaaattcggttaaggccaggggaaagaaaaatataaattaa
aacatatagtatgggcaagcagggagctagaacgattcgcagttaatcctggcctgttagaaacatcagaaggctgtagacaaatactg
ggacagctacaaccatcccttcagacaggatcagaagaacttagatcattatataatacagtagcaaccctctattgtgtgcatcaaagg
atagagataaaagacaccaaggaagctttagacaagatagaggaagagcaaaacaaaagtaagaccaccgcacagcaagcggccg
ctgatcttcagacctggaggaggagatatgagggacaattggagaagtgaattatataaatataaagtagtaaaaattgaaccattagga
gtagcacccaccaaggcaaagagaagagtggtgcagagagaaaaaagagcagtgggaataggagctttgttccttgggttcttggga
gcagcaggaagcactatgggcgcagcctcaatgacgctgacggtacaggccagacaattattgtctggtatagtgcagcagcagaac
aatttgctgagggctattgaggcgcaacagcatctgttgcaactcacagtctggggcatcaagcagctccaggcaagaatcctggctgt
ggaaagatacctaaaggatcaacagctcctggggatttggggttgctctggaaaactcatttgcaccactgctgtgccttggaatgctag
ttggagtaataaatctctggaacagattggaatcacacgacctggatggagtgggacagagaaattaacaattacacaagcttaatacac
tccttaattgaagaatcgcaaaaccagcaagaaaagaatgaacaagaattattggaattagataaatgggcaagtttgtggaattggttta
acataacaaattggctgtggtatataaaattattcataatgatagtaggaggcttggtaggtttaagaatagtttttgctgtactttctatagtg
aatagagttaggcagggatattcaccattatcgtttcagacccacctcccaaccccgaggggacccgacaggcccgaaggaatagaa

gaagaaggtggagagagagacagagacagatccattcgattagtgaacggatctcgacggtatcggttaacttttaaaagaaaaggg
gggattgggggtacagtgcaggggaaagaatagtagacataatagcaacagacatacaaactaaagaattacaaaaacaaattaca
aaaattcaaaattttccgatcacgagactagcctcgaggtcgacattgattattgactagttattaatagtaatcaattacggggtcattagtt
catagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtc
aataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgggtggactatttacggtaaactgcccacttggca
gtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcctggcattatgcccagtacatgacc
ttatgggactttcctacttggcagtacatctacgtattagtcatcgctattaccatgggtcgaggtgagccccacgttctgcttcactctccc
catctccccccccctccccacccccaattttgtatttatttatttttttaattattttgtgcagcgatgggggcgggggggggggggggcgcgcg
ccaggcggggcggggcggggcgagggggcggggcggggcgaggcggagaggtgcggcggcagccaatcagagcggcgcgct
ccgaaagtttccttttatggcgaggcggcggcggcggcccctataaaaagcgaagcgcgcggcgggcgggagtcgctgcgttg
ccttcgccccgtgccccgctccgcgccgcctcgcgccgcccgccccggctctgactgaccgcgttactcccacaggtgagcgggcg
ggacggcccttctcctccgggctgtaattagcgcttggtttaatgacggctcgtttcttttctgtggctgcgtgaaagccttaaagggctcc
gggagggcccttttgtgcgggggggagcggctcgggggtgcgtgcgtgtgtgtgtgcgtggggagcgccgcgtgcggcccgcgc
tgcccggcggctgtgagcgctgcgggcgcggcgcggggctttgtgcgctccgcgtgtgcgcgaggggagcgcggccgggggcg
gtgccccgcggtgcgggggggctgcgaggggaacaaaggctgcgtgcggggtgtgtgcgtgggggggtgagcaggggtgtgg
gcgcggcggtcgggctgtaacccccccctgcacccccctccccgagttgctgagcacggcccggcttcgggtgcggggctccgtgc
ggggcgtggcgcggggctcgccgtgccgggcgggggggtggcggcaggtgggggtgccgggcggggcggggccgcctcgggc
cggggagggctcgggggaggggcgcggcggccccggagcgccggcggctgtcgaggcgcggcgagccgcagccattgccttttt
atggtaatcgtgcgagagggcgcagggacttcctttgtcccaaatctggcggagccgaaatctgggaggcgccgccgcacccccctct
agcgggcgcgggcgaagcggtgcggcgccggcaggaaggaaatgggcggggagggccttcgtgcgtcgccgcgccgccgtcc
ccttctccatctccagcctcggggctgccgcaggggacggctgccttcgggggggacggggcagggcggggttcggcttctggc
gtgtgaccggcggctctagagcctctgctaaccatgttcatgccttcttcttttttcctacagctcctgggcaacgtgctggttattgtgctgtc
tcatcattttggcaaagaattatcgcgatcgccatgaccgagtacaagcccacggtgcgcctcgccacccgcgacgacgtccccagg
gccgtacgcaccctcgccgccgcgttcgccgactaccccgccacgcgccacaccgtcgatccggaccgccacatcgagcgggtca
ccgagctgcaagaactcttcctcacgcgcgtcgggctcgacatcggcaaggtgtgggtcgcggacgacggcgccgcggtggcggt
ctggaccacgccggagagcgtcgaagcgggggcggtgttcgccgagatcggcccgcgcatggccgagttgagcggttcccggctg
gccgcgcagcaacagatggaaggcctcctggcgccgcaccggcccaaggagcccgcgtggttcctggccaccgtcggcgtctcgc
ccgaccaccagggcaagggtctgggcagcgccgtcgtgctccccggagtggaggcggccgagcgcgccggggtgcccgccttcc
tggagacctccgcgccccgcaacctccccttctacgagcggctcggcttcaccgtcaccgccgacgtcgaggtgcccgaaggaccg
cgcacctggtgcatgacccgcaagcccggtgccacgcgtcagtgtactaattatgctctcttgaaattggctggagatgttgagagcaa
cccaggtcccatggaagacgccaaaaacataaagaaaggcccggcgccattctatccgctggaagatggaaccgctggagagcaa
ctgcataaggctatgaagagatacgccctggttcctggaacaattgcttttacagatgcacatatcgaggtggacatcacttacgctgagt
acttcgaaatgtccgttcggttggcagaagctatgaaacgatatgggctgaatacaaatcacagaatcgtcgtatgcagtgaaaactctct
tcaattctttatgccggtgttgggcgcgttatttatcggagttgcagttgcgcccgcgaacgacatttataatgaacgtgaattgctcaaca

gtatgggcatttcgcagcctaccgtggtgttcgtttccaaaaaggggttgcaaaaaattttgaacgtgcaaaaaaagctcccaatcatcca
aaaaattattatcatggattctaaaacggattaccagggatttcagtcgatgtacacgttcgtcacatctcatctacctcccggttttaatgaa
tacgattttgtgccagagtccttcgatagggacaagacaattgcactgatcatgaactcctctggatctactggtctgcctaaaggtgtcgc
tctgcctcatagaactgcctgcgtgagattctcgcatgccagagatcctatttttggcaatcaaatcattccggatactgcgattttaagtgtt
gttccattccatcacggttttggaatgtttactacactcggatatttgatatgtggatttcgagtcgtcttaatgtatagatttgaagaagagct
gtttctgaggagccttcaggattacaagattcaaagtgcgctgctggtgccaaccctattctccttcttcgccaaaagcactctgattgaca
aatacgatttatctaatttacacgaaattgcttctggtggcgctcccctctctaaggaagtcggggaagcggttgccaagaggttccatct
gccaggtatcaggcaaggatatgggctcactgagactacatcagctattctgattacacccgagggggatgataaaccgggcgcggtc
ggtaaagttgttccatttttgaagcgaaggttgtggatctggataccgggaaaacgctgggcgttaatcaaagaggcgaactgtgtgtg
agaggtcctatgattatgtccggttatgtaaacaatccggaagcgaccaacgccttgattgacaaggatggatggctacattctggagac
atagcttactgggacgaagacgaacacttcttcatcgttgaccgcctgaagtctctgattaagtacaaaggctatcaggtggctcccgct
gaattggaatccatcttgctccaacaccccaacatcttcgacgcaggtgtcgcaggtcttcccgacgatgacgccggtgaacttcccgc
cgccgttgttgttttggagcacggaaagacgatgacggaaaaagagatcgtggattacgtcgccagtcaagtaacaaccgcgaaaaa
gttgcgcggaggagttgtgtttgtggacgaagtaccgaaaggtcttaccggaaaactcgacgcaagaaaaatcagagagatcctcata
aaggccaagaagggcggaaagatcgccgtgtaagtttaaacgtcgacaatcaacctctggattacaaaatttgtgaaagattgactggt
attcttaactatgttgctccttttacgctatgtggatacgctgctttaatgcctttgtatcatgctattgcttcccgtatggctttcattttctcctcct
tgtataaatcctggttgctgtctctttatgaggagttgtggcccgttgtcaggcaacgtggcgtggtgtgcactgtgtttgctgacgcaacc
cccactggttggggcattgccaccacctgtcagctcctttccgggactttcgctttcccccctccctattgccacggcggaactcatcgccg
cctgccttgcccgctgctggacaggggctcggctgttgggcactgacaattccgtggtgttgtcggggaagctgacgtcctttccatgg
ctgctcgcctgtgttgccacctggattctgcgcgggacgtccttctgctacgtcccttcggccctcaatccagcggaccttccttcccgcg
gcctgctgccggctctgcggcctcttccgcgtcttcgccttcgccctcagacgagtcggatctccctttgggccgcctccccgcctgga
attcgagctcggtacctttaagaccaatgacttacaaggcagctgtagatcttagccacttttaaaagaaaaggggggactggaaggg
ctaattcactcccaacgaagacaagatctgctttttgcttgtactgggtctctctggttagaccagatctgagcctgggagctctctggcta
actagggaacccactgcttaagcctcaataaagcttgccttgagtgcttcaagtagtgtgtgcccgtctgttgtgtgactctggtaactaga
gatccctcagacccttttagtcagtgtggaaaatctctagcagtagtagttcatgtcatcttattattcagtatttataacttgcaaagaaatga
atatcagagagtgagaggaacttgtttattgcagcttataatggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttt
cactgcattctagttgtggtttgtccaaactcatcaatgtatcttatcatgtctggctctagctatcccgcccctaactccgcccagttccgcc
cattctccgccccatggctgactaatttttttttatttatgcagaggccgaggccgcctcggcctctgagctattccagaagtagtgaggag
gctttttttggaggcctaggcttttgcgtcgagacgtacccaattcgccctatagtgagtcgtattacgcgcgctcactggccgtcgttttac
aacgtcgtgactgggaaaaccctggcgttacccaacttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagag
gcccgcaccgatcgcccttcccaacagttgcgcagcctgaatggcgaatggcgcgacgcgccctgtagcggcgcattaagcgcggc
gggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgccctagcgcccgctcctttcgctttcttcccttcctttctcgccacg
ttcgccggctttccccgtcaagctctaaatcgggggctccctttagggttccgatttagtgctttacggcacctcgaccccaaaaaacttg
attagggtgatggttcacgtagtgggccatcgccctgatagacggtttttcgccctttgacgttggagtccacgttctttaatagtggactct

tgttccaaactggaacaacactcaaccctatctcggtctattcttttgatttataagggattttgccgatttcggcctattggttaaaaaatgag
ctgatttaacaaaaatttaacgcgaattttaacaaaatattaacgtttacaatttcc

S3

pEV EF1-c-myc sequence

gctagcggctccggtgcccgtcagtgggcagagcgcacatcgcccacagtccccgagaagttgggggggaggggtcggcaattgaa
ccggtgcctagagaaggtggcgcggggtaaactgggaaagtgatgtcgtgtactggctccgccttttttcccgagggtgggggagaac
cgtatataagtgcagtagtcgccgtgaacgttctttttcgcaacgggtttgccgccagaacacaggtaagtgccgtgtgtggttcccgcg
ggcctggcctctttacgggttatggcccttgcgtgccttgaattacttccactggctgcagtacgtgattcttgatcccgagcttcgggttg
gaagtgggtgggagagttcgaggccttgcgcttaaggagcccttcgcctcgtgcttgagttgaggcctggcctgggcgctggggcc
gccgcgtgcgaatctggtggcaccttcgcgcctgtctcgctgctttcgataagtctctagccatttaaaatttttgatgacctgctgcgacg
ctttttttctggcaagatagtcttgtaaatgcgggccaagatctgcacactggtatttcggttttggggccgcgggcggcgacggggcccc
gtgcgtcccagcgcacatgttcggcgaggcggggcctgcgagcgcggccaccgagaatcggacgggggtagtctcaagctggcc
ggcctgctctggtgcctggcctcgcgccgccgtgtatcgccccgccctgggcggcaaggctggcccggtcggcaccagttgcgtga
gcggaaagatggccgcttcccggccctgctgcagggagctcaaaatggaggacgcggcgctcgggagagcgggcgggtgagtca
cccacacaaaggaaaagggcctttccgtcctcagccgtcgcttcatgtgactccacggagtaccgggcgccgtccaggcacctcgatt
agttctcgagcttttggagtacgtcgtctttaggttgggggggaggggttttatgcgatggagtttccccacactgagtgggtggagactga
agttaggccagcttggcacttgatgtaattctccttggaatttgccctttttgagtttggatcttggttcattctcaagcctcagacagtggttc
aaagttttttttcttccatttcaggtgtcgtgagcgatcgccatggatttttttcgggtagtggaaaaccagcagcctcccgcgacgatgccc
ctcaacgttagcttcaccaacaggaactatgacctcgactacgactcggtgcagccgtatttctactgcgacgaggaggagaacttctac
cagcagcagcagcagagcgagctgcagcccccggcgcccagcgaggatatctggaagaaattcgagctgctgcccaccccgccc
ctgtcccctagccgccgctccgggctctgctcgccctcctacgttgcggtcacacccttctcccttcggggagacaacgacggcggtg
gcgggagcttctccacggccgaccagctggagatggtgaccgagctgctgggaggagacatggtgaaccagagtttcatctgcgac
ccggacgacgagaccttcatcaaaaacatcatcatccaggactgtatgtggagcggcttctcggccgccgccaagctcgtctcagaga
agctggcctcctaccaggctgcgcgcaaagacagcggcagcccgaaccccgcccgcggccacagcgtctgctccacctccagctt
gtacctgcaggatctgagcgccgccgcctcagagtgcatcgacccctcggtggtcttcccctaccctctcaacgacagcagctcgccc
aagtcctgcgcctcgcaagactccagcgccttctctccgtcctcggattctctgctctcctcgacggagtcctccccgcagggcagccc
cgagcccctggtgctccatgaggagacaccgcccaccaccagcagcgactctgaggaggaacaagaagatgaggaagaaatcgat
gttgtttctgtggaaaagaggcaggctcctggcaaaaggtcagagtctggatcaccttctgctggaggccacagcaaacctcctcacag
cccactggtcctcaagaggtgccacgtctccacacatcagcacaactacgcagcgcctccctccactcggaaggactatcctgctgcc
aagagggtcaagttggacagtgtcagagtcctgagacagatcagcaacaaccgaaaatgcaccagccccaggtcctcggacaccga
ggagaatgtcaagaggcgaacacacaacgtcttggagcgccagaggaggaacgagctaaaacggagctttttgccctgcgtgacc

agatcccggagttggaaaacaatgaaaaggcccccaaggtagttatccttaaaaaagccacagcatacatcctgtccgtccaagcaga
ggagcaaaagctcatttctgaagaggacttgttgcggaaacgacgagaacagttgaaacacaaacttgaacagctacggaactcttgt
gcgacgcgtacgcggccgctcgagcagaaactcatctcagaagaggatctggcagcaaatgatatcctgggaggcggaggcagcc
gcagacgtcgccggagaagacgccgccgcagataagtttaaacgcgtcgacaatcaacctctggattacaaaatttgtgaaagattga
ctggtattcttaactatgttgctccttttacgctatgtggatacgctgctttaatgcctttgtatcatgctattgcttcccgtatggctttcattttct
cctccttgtataaatcctggttgctgtctctttatgaggagttgtggcccgttgtcaggcaacgtggcgtggtgtgcactgtgtttgctgacg
caacccccactggttggggcattgccaccacctgtcagctcctttccgggactttcgctttccccctccctattgccacggcggaactcat
cgccgcctgccttgcccgctgctggacaggggctcggctgttgggcactgacaattccgtggtgttgtcggggaagctgacgtcctttc
catggctgctcgcctgtgttgccacctggattctgcgcgggacgtccttctgctacgtcccttcggccctcaatccagcggaccttccttc
ccgcggcctgctgccggctctgcggcctcttccgcgtcttcgccttcgccctcgacgagtcggatctccctttgggcggatccagacat
gataagatacattgatgagtttggacaaaccacaactagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttg
taaccattataagctgcaataaacaagttaacaacaacaattgcattcattttatgtttcaggttcaggggaggtgtgggaggttttttaaa
gcaagtaaaacctctacaaatgtggtatggctgattatgatccggctgcctcgcgcgtttcggtgatgacggtgaaaacctctgacacat
gcagctcccggagacggtcacagcttgtctgtaagcggatgccgggagcagacaagcccgtcaggcgtcagcgggtgttggcgggt
gtcggggcgcagccatgaggtcgactctagaggatcgatgccccgccccggacgaactaaacctgactacgacatctctgccccttct
tcgcggggcagtgcatgtaatcccttcagttggttggtacaacttgccaactgggccctgttccacatgtgacacgggggggaccaaa
cacaaaggggttctctgactgtagttgacatccttataaatggatgtgcacatttgccaacactgagtggctttcatcctggagcagacttt
gcagtctgtggactgcaacacaacattgcctttatgtgtaactcttggctgaagctcttacaccaatgctgggggacatgtacctcccagg
ggcccaggaagactacgggaggctacaccaacgtcaatcagaggggcctgtgtagctaccgataagcggaccctcaagagggcatt
agcaatagtgtttataaggccccttgttaaccctaaacgggtagcatatgcttcccgggtagtagtatatactatccagactaaccctaatt
caatagcatatgttacccaacgggaagcatatgctatcgaattagggttagtaaaagggtcctaaggaacagcgatatctcccaccccat
gagctgtcacggttttatttacatggggtcaggattccacgagggtagtgaaccattttagtcacaagggcagtggctgaagatcaagga
gcgggcagtgaactctcctgaatcttcgcctgcttcttcattctccttcgtttagctaatagaataactgctgagttgtgaacagtaaggtgta
tgtgaggtgctcgaaaacaaggtttcaggtgacgccccagaataaaatttggacgggggttcagtggtggcattgtgctatgacacc
aatataaccctcacaaacccctttgggcaataaatactagtgtaggaatgaaacattctgaatatctttaacaatagaaatccatggggtgg
ggacaagccgtaaagactggatgtccatctcacacgaatttatggctatgggcaacacataatcctagtgcaatatgatactggggttatt
aagatgtgtcccaggcagggaccaagacaggtgaaccatgttgttacactctatttgtaacaaggggaaagagagtggacgccgaca
gcagcggactccactggttgtctctaacacccccgaaaattaaacggggctccacgccaatggggcccataaacaaagacaagtggc
cactctttttttttgaaattgtggagtgggggcacgcgtcagcccccacacgccgccctgcggttttggactgtaaaataagggtgtaataa
cttggctgattgtaaccccgctaaccactgcggtcaaaccacttgcccacaaaaccactaatggcaccccggggaatacctgcataagt
aggtgggcgggccaagataggggcgcgattgctgcgatctggaggacaaattacacacacttgcgcctgagcgccaagcacagggt
tgttggtcctcatattcacgaggtcgctgagagcacggtgggctaatgttgccatgggtagcatatactacccaaatatctggatagcata
tgctatcctaatctatatctgggtagcataggctatcctaatctatatctgggtagcatatgctatcctaatctatatctgggtagtatatgctat
cctaatttatatctgggtagcataggctatcctaatctatatctgggtagcatatgctatcctaatctatatctgggtagtatatgctatcctaat

ctgtatccgggtagcatatgctatcctaatagagattagggtagtatatgctatcctaatttatatctgggtagcatatactacccaaatatct
ggatagcatatgctatcctaatctatatctgggtagcatatgctatcctaatctatatctgggtagcataggctatcctaatctatatctgggta
gcatatgctatcctaatctatatctgggtagtatatgctatcctaatttatatctgggtagcataggctatcctaatctatatctgggtagcatat
gctatcctaatctatatctgggtagtatatgctatcctaatctgtatccgggtagcatatgctatcctcatgcatatacagtcagcatatgatac
ccagtagtagagtgggagtgctatcctttgcatatgccgccacctcccaaggggcgtgaattttcgctgcttgtccttttcctgctggttg
ctcccattcttaggtgaatttaaggaggccaggctaaagccgtcgcatgtctgattgctcaccaggtaaatgtcgctaatgttttccaacgc
gagaaggtgttgagcgcggagctgagtgacgtgacaacatgggtatgcccaattgccccatgttgggaggacgaaaatggtgacaag
acagatggccagaaatacaccaacagcacgcatgatgtctactggggatttattctttagtgcgggggaatacacggcttttaatacgatt
gagggcgtctcctaacaagttacatcactcctgcccttcctcaccctcatctccatcacctccttcatctccgtcatctccgtcatcaccctc
cgcggcagccccttccaccataggtggaaaccagggaggcaaatctactccatcgtcaaagctgcacacagtcaccctgatattgcag
gtaggagcgggctttgtcataacaaggtccttaatcgcatccttcaaaacctcagcaaatatatgagtttgtaaaaagaccatgaaataac
agacaatggactcccttagcgggccaggttgtgggccgggtccaggggccattccaaaggggagacgactcaatggtgtaagacga
cattgtggaatagcaagggcagttcctcgccttaggttgtaaagggaggtcttactacctccatatacgaacacaccggcgacccaagtt
ccttcgtcggtagtcctttctacgtgactcctagccaggagagctcttaaaccttctgcaatgttctcaaatttcgggttggaacctccttga
ccacgatgctttccaaaccaccctccttttttgcgcctgcctccatcaccctgaccccggggtccagtgcttgggccttctcctgggtcatc
tgcggggccctgctctatcgctcccgggggcacgtcaggctcaccatctgggccaccttcttggtggtattcaaaataatcggcttcccc
tacagggtggaaaaatggccttctacctggaggggcctgcgcggtggagacccggatgatgatgactgactactgggactcctggg
cctcttttctccacgtccacgacctctccccctggctctttcacgacttcccccctggctctttcacgtcctctaccccggcggcctccact
acctcctcgaccccggcctccactacctcctcgaccccggcctccactgcctcctcgaccccggcctccacctcctgctcctgccccctc
ctgctcctgccccctcctcctgctcctgccccctcctgccccctcctgctcctgccccctcctgccccctcctgctcctgccccctcctgccccctcct
gctcctgccccctcctgccccctcctcctgctcctgccccctcctgccccctcctcctgctcctgccccctcctgctcctgccccctcctgccccctcc
tgccccctcctgctcctgccccctcctgccccctcctgctcctgccccctcctgctcctgccccctcctgctcctgccccctcctgctcctgccccctc
ctgccccctcctgccccctcctcctgctcctgccccctcctgctcctgccccctcctgccccctcctgccccctcctgctcctgccccctcctcctgct
cctgccccctcctgccccctcctgccccctcctcctgctcctgccccctcctgccccctcctcctgctcctgccccctcctcctgctcctgccccctcc
tgccccctcctgccccctcctcctgctcctgccccctcctgccccctcctcctgctcctgccccctcctcctgctcctgccccctcctcctgctcctg
cccctcctcctgctcctgccccctcctcctgctcctgccccctcctgccccctcctgccccctcctgccccctcctgctcctgccccctcctcctcct
gctcctgccccctcctgctcctgccccctcccgctcctgctcctgctcctgttccaccgtgggtccccttgcagccaatgcaacttggacgttt
ttggggtctccggacaccatctctatgtcttggccctgatcctgagccgcccggggctcctggtcttccgcctcctcgtcctcgtcctcttc
cccgtcctcgtccatggttatcacccccctcttctttgaggtccactgccgccggagccttctggtccagatgtgtctcccttctctcctaggc
catttccaggtcctgtacctggcccctcgtcagacatgattcacactaaaagagatcaatagacatctttattagacgacgctcagtgaata
cagggagtgcagactcctgccccctccaacagccccccacccctcatccccttcatggtcgctgtcagacagatccaggtctgaaaatt
ccccatcctccgaaccatcctcgtcctcatcaccaattactcgcagcccggaaaactcccgctgaacatcctcaagatttgcgtcctgag
cctcaagccaggcctcaaattcctcgtcccccttttgctggacggtagggatggggattctcgggacccctcctcttcctcttcaaggtc
accagacagagatgctactggggcaacggaagaaaagctgggtgcggcctgtgaggatcagcttatcgatgataagctgtcaaacat

gagaattcttgaagacgaaagggcctcgtgatacgcctatttttataggttaatgtcatgataataatggtttcttagacgtcaggtggcact
tttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgct
tcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattccctttttgcggcattttgccttcctgtttttgctcac
ccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctcaacagcggtaaga
tccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggtattatcccgtgttgacgccg
ggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttacggatggc
atgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcggaggaccgaa
ggagctaaccgcttttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccataccaaacg
acgagcgtgacaccacgatgcctgcagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccggcaa
caattaatagactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttattgctgataaatctg
gagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttatctacacgacggg
gagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaagttta
ctcatatatactttagattgatttaaaacttcatttttaatttaaaaggatctaggtgaagatccttttgataatctcatgaccaaaatcccttaac
gtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatccttttttctgcgcgtaatctgctgcttgca
aacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagc
gcagataccaaatactgtccttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgcta
atcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcg
gtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctat
gagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagg
gagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcagg
ggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttgctggccttgaagctgtccctgatggtcgtc
atctacctgcctggacagcatggcctgcaacgcgggcatcccgatgccgccggaagcgagaagaatcataatggggaaggccatcc
agcctcgcgtcgcga

S4

pev ef1-Oct4-E2A-Sox2-E2A-Klf4 sequence

gctagcggctccggtgcccgtcagtgggcagagcgcacatcgcccacagtccccgagaagttgggggggaggggtcggcaattgaa
ccggtgcctagagaaggtggcgcggggtaaactgggaaagtgatgtcgtgtactggctccgccttttcccgagggtgggggagaac
cgtatataagtgcagtagtcgccgtgaacgttctttttcgcaacgggtttgccgccagaacacaggtaagtgccgtgtgtggttcccgcg
ggcctggcctctttacgggttatggcccttgcgtgccttgaattacttccactggctgcagtacgtgattcttgatcccgagcttcgggttg

gaagtgggtgggagagttcgaggccttgcgcttaaggagccccttcgcctcgtgcttgagttgaggcctggcctgggcgctggggcc
gccgcgtgcgaatctggtggcaccttcgcgcctgtctcgctgctttcgataagtctctagccatttaaaatttttgatgacctgctgcgacg
ctttttttctggcaagatagtcttgtaaatgcgggccaagatctgcacactggtatttcggttttggggccgcgggcggcgacggggccc
gtgcgtcccagcgcacatgttcggcgaggcggggcctgcgagcgcggccaccgagaatcggacgggggtagtctcaagctggcc
ggcctgctctggtgcctggcctcgcgccgccgtgtatcgccccgccctgggcggcaaggctggcccggtcggcaccagttgcgtga
gcggaaagatggccgcttcccggccctgctgcagggagctcaaaatggaggacgcggcgctcgggagagcgggcgggtgagtca
cccacacaaaggaaaagggcctttccgtcctcagccgtcgcttcatgtgactccacggagtaccgggcgccgtccaggcacctcgatt
agttctcgagcttttggagtacgtcgtctttaggttgggggggaggggttttatgcgatggagtttccccacactgagtgggtggagactga
agttaggccagcttggcacttgatgtaattctccttggaatttgccctttttgagtttggatcttggttcattctcaagcctcagacagtggttc
aaagtttttttcttccatttcaggtgtcgtgagcgatcgccatggcgggacacctggcttcggatttcgccttctcgccccctccaggtggt
ggaggtgatgggccaggggggccggagccgggctgggttgatcctcggacctggctaagcttccaaggccctcctggagggccag
gaatcgggccgggggttgggccaggctctgaggtgtgggggattcccccatgccccccgccgtatgagttctgtgggggggatggcgt
actgtgggccccaggttggagtggggctagtgccccaaggcggcttggagacctctcagcctgagggcgaagcaggagtcggggt
ggagagcaactccgatggggcctccccggagccctgcaccgtcacccctggtgccgtgaagctggagaaggagaagctggagcaa
aacccggaggagtcccaggacatcaaagctctgcagaaagaactcgagcaatttgccaagctcctgaagcagaagaggatcaccct
gggatatacacaggccgatgtggggctcaccctgggggttctatttgggaaggtattcagccaaacgaccatctgccgctttgaggctc
tgcagcttagcttcaagaacatgtgtaagctgcggcccttgctgcagaagtgggtggaggaagctgacaacaatgaaaatcttcagga
gatatgcaaagcagaaaccctcgtgcaggcccgaaagagaaagcgaaccagtatcgagaaccgagtgagaggcaacctggagaat
ttgttcctgcagtgcccgaaacccacactgcagcagatcagccacatcgcccagcagcttgggctcgagaaggatgtggtccgagtgt
ggttctgtaaccggcgccagaagggcaagcgatcaagcagcgactatgcacaacgagaggattttgaggctgctgggtctccttttctc
agggggaccagtgtcctttcctctggccccagggccccattttggtaccccaggctatgggagccctcacttcactgcactgtactcctc
ggtccctttccctgaggggggaagcctttcccccctgtctccgtcaccactctgggctctcccatgcattcaaaccagtgtactaattatgctc
tcttgaaattggctggagatgttgagagcaacccaggtcccatgtacaacatgatggagacggagctgaagccgccgggcccgcagc
aaacttcgggggggcggcggcggcaactccaccgcggcggcggccggcggcaaccagaaaaacagcccggaccgcgtcaagcg
gcccatgaatgccttcatggtgtggtcccgcgggcagcggcgcaagatggcccaggagaaccccaagatgcacaactcggagatca
gcaagcgcctgggcgccgagtggaaacttttgtcggagacggagaagcggccgttcatcgacgaggctaagcggctgcgagcgct
gcacatgaaggagcacccggattataaataccggccccggcggaaaaccaagacgctcatgaagaaggataagtacacgctgccc
ggcgggctgctggcccccggcggcaatagcatggcgagcggggtcggggtgggcgccggcctgggcgcgggcgtgaaccagc
gcatggacagttacgcgcacatgaacggctggagcaacggcagctacagcatgatgcaggaccagctgggctacccgcagcaccc
gggcctcaatgcgcacggcgcagcgcagatgcagcccatgcaccgctacgacgtgagcgccctgcagtacaactccatgaccagct
cgcagacctacatgaacggctcgcccacctacagcatgtcctactcgcagcagggcacccctggcatggctcttggctccatgggttc
ggtggtcaagtccgaggccagctccagccccctgtggttacctcttcctcccactccagggcgccctgccaggccggggacctccg
ggacatgatcagcatgtatctccccggcgccgaggtgccggaacccgccgcccccagcagacttcacatgtcccagcactaccaga
gcggcccggtgcccggcacggccattaacggcacactgcccctctcacacatgacgcgtcaatgtactaactacgctttgttgaaactc

gctggcgatgttgaaagtaaccccggtcctatgaggcagccacctggcgagtctgacatggctgtcagcgacgcgctgctcccatcttt
ctccacgttcgcgtctggcccggcgggaagggagaagacactgcgtcaagcaggtgccccgaataaccgctggcgggaggagctc
tcccacatgaagcgacttcccccagtgcttcccggccgcccctatgacctggcggcggcgaccgtggccacagacctggagagcgg
cggagccggtgcggcttgcggcggtagcaacctggcgcccctacctcggagagagaccgaggagttcaacgatctcctggacctgg
actttattctctccaattcgctgacccatcctccggagtcagtggccgccaccgtgtcctcgtcagcgtcagcctcctcttcgtcgtcgccg
tcgagcagcggccctgccagcgcgccctccacctgcagcttcacctatccgatccgggccgggaacgacccgggcgtggcgccgg
gcggcacgggcggaggcctcctctatggcagggagtccgctccccctccgacggctcccttcaacctggcggacatcaacgacgtg
agcccctcgggcggcttcgtggccgagctcctgcggccagaattggacccggtgtacattccgccgcagcagccgcagccgccag
gtggcgggctgatgggcaagttcgtgctgaaggcgtcgctgagcgcccctggcagcgagtacggcagcccgtcggtcatcagcgtc
agcaaaggcagccctgacggcagccacccggtggtggtggcgccctacaacggcgggccgccgcgcacgtgccccaagatcaag
caggaggcggtctcttcgtgcacccacttgggcgctggacccccctctcagcaatggccaccggccggctgcacacgacttcccccctg
gggcggcagctccccagcaggactaccccgaccctgggtcttgaggaagtgctgagcagcagggactgtcaccctgccctgccgct
tcctcccggcttccatccccacccggggcccaattacccatccttcctgcccgatcagatgcagccgcaagtcccgccgctccattacc
aagagctcatgccacccggttcctgcatgccagaggagcccaagccaaagaggggaagacgatcgtggccccggaaaaggaccg
ccacccacacttgtgattacgcgggctgcggcaaaacctacacaaagagttcccatctcaaggcacacctgcgaacccacacaggtg
agaaaccttaccactgtgactgggacggctgtggatggaaattcgcccgctcagatgaactgaccaggcactaccgtaaacacacgg
ggcaccgcccgttccagtgccaaaaatgcgaccgagcattttccaggtcggaccacctcgccttacacatgaagaggcatttttaagttt
aaacgcgtcgacaatcaacctctggattacaaaatttgtgaaagattgactggtattcttaactatgttgctccttttacgctatgtggatacg
ctgctttaatgcctttgtatcatgctattgcttcccgtatggctttcattttctcctccttgtataaatcctggttgctgtctctttatgaggagttgt
ggcccgttgtcaggcaacgtggcgtggtgtgcactgtgtttgctgacgcaacccccactggttggggcattgccaccacctgtcagctc
ctttccgggactttcgctttcccccctccctattgccacggcggaactcatcgccgcctgccttgcccgctgctggacaggggctcggctg
ttgggcactgacaattccgtggtgttgtcggggaagctgacgtcctttccatggctgctcgcctgtgttgccacctggattctgcgcggga
cgtccttctgctacgtcccttcggccctcaatccagcggaccttccttcccgcggcctgctgccggctctgcggcctcttccgcgtcttcg
ccttcgccctcgacgagtcggatctccctttgggcggatccagacatgataagatacattgatgagtttggacaaaccacaactagaatg
cagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaaccattataagctgcaataaacaagttaacaacaacaattg
cattcattttatgtttcaggttcagggggaggtgtgggaggttttttaaagcaagtaaaacctctacaaatgtggtatggctgattatgatcc
ggctgcctcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgtaagcggatg
ccgggagcagacaagcccgtcaggcgtcagcgggtgttggcgggtgtcggggcgcagccatgaggtcgactctagaggatcgatg
ccccgccccggacgaactaaacctgactacgacatctctgccccttcttcgcggggcagtgcatgtaatcccttcagttggttggtacaa
cttgccaactgggccctgttccacatgtgacacgggggggaccaaacacaaaggggttctctgactgtagttgacatccttataaatg
gatgtgcacatttgccaacactgagtggctttcatcctggagcagactttgcagtctgtggactgcaacacaacattgcctttatgtgtaac
tcttggctgaagctcttacaccaatgctgggggacatgtacctcccaggggcccaggaagactacgggaggctacaccaacgtcaatc
agaggggcctgtgtagctaccgataagcggaccctcaagagggcattagcaatagtgtttataaggccccttgttaaccctaaacggg
tagcatatgcttcccgggtagtagtatatactatccagactaaccctaattcaatagcatatgttacccaacgggaagcatatgctatcgaa

ttagggttagtaaaagggtcctaaggaacagcgatatctcccaccccatgagctgtcacggttttatttacatggggtcaggattccacga
gggtagtgaaccattttagtcacaagggcagtggctgaagatcaaggagcgggcagtgaactctcctgaatcttcgcctgcttcttcatt
ctccttcgtttagctaatagaataactgctgagttgtgaacagtaaggtgtatgtgaggtgctcgaaaacaaggtttcaggtgacgccccc
agaataaaatttggacgggggttcagtggtggcattgtgctatgacaccaatataaccctcacaaaccccttgggcaataaatactagt
gtaggaatgaaacattctgaatatctttaacaatagaaatccatggggtggggacaagccgtaaagactggatgtccatctcacacgaat
ttatggctatgggcaacacataatcctagtgcaatatgatactggggttattaagatgtgtcccaggcagggaccaagacaggtgaacc
atgttgttacactctatttgtaacaaggggaaagagagtggacgccgacagcagcggactccactggttgtctctaacacccccgaaaa
ttaaacggggctccacgccaatggggcccataaacaaagacaagtggccactctttttttgaaattgtggagtgggggcacgcgtcag
cccccacacgccgccctgcggttttggactgtaaaataagggtgtaataacttggctgattgtaaccccgctaaccactgcggtcaaacc
acttgcccacaaaaccactaatggcaccccggggaatacctgcataagtaggtgggcgggccaagataggggcgcgattgctgcga
tctggaggacaaattacacacacttgcgcctgagcgccaagcacagggttgttggtcctcatattcacgaggtcgctgagagcacggt
gggctaatgttgccatgggtagcatatactacccaaatatctggatagcatatgctatcctaatctatatctgggtagcataggctatcctaa
tctatatctgggtagcatatgctatcctaatctatatctgggtagtatatgctatcctaatttatatctgggtagcataggctatcctaatctatat
ctgggtagcatatgctatcctaatctatatctgggtagtatatgctatcctaatctgtatccgggtagcatatgctatcctaatagagattagg
gtagtatatgctatcctaatttatatctgggtagcatatactacccaaatatctggatagcatatgctatcctaatctatatctgggtagcatat
gctatcctaatctatatctgggtagcataggctatcctaatctatatctgggtagcatatgctatcctaatctatatctgggtagtatatgctatc
ctaatttatatctgggtagcataggctatcctaatctatatctgggtagcatatgctatcctaatctatatctgggtagtatatgctatcctaatc
tgtatccgggtagcatatgctatcctcatgcatatacagtcagcatatgatacccagtagtagagtgggagtgctatcctttgcatatgccg
ccacctcccaaggggggcgtgaattttcgctgcttgtccttttcctgctggttgctcccattcttaggtgaatttaaggaggccaggctaaag
ccgtcgcatgtctgattgctcaccaggtaaatgtcgctaatgttttccaacgcgagaaggtgttgagcgcggagctgagtgacgtgaca
acatgggtatgcccaattgccccatgttgggaggacgaaaatggtgacaagacagatggccagaaatacaccaacagcacgcatgat
gtctactggggatttattctttagtgcgggggaatacacggcttttaatacgattgagggcgtctcctaacaagttacatcactcctgcccctt
cctcaccctcatctccatcacctccttcatctccgtcatctccgtcatcaccctccgcggcagccccttccaccataggtggaaaccaggg
aggcaaatctactccatcgtcaaagctgcacacagtcaccctgatattgcaggtaggagcgggctttgtcataacaaggtccttaatcgc
atccttcaaaacctcagcaaatatatgagtttgtaaaaagaccatgaaataacagacaatggactcccttagcgggccaggttgtgggcc
gggtccaggggccattccaaaggggagacgactcaatggtgtaagacgacattgtggaatagcaagggcagttcctcgccttaggttg
taaagggaggtcttactacctccatatacgaacacaccggcgacccaagttccttcgtcggtagtcctttctacgtgactcctagccagg
agagctcttaaaccttctgcaatgttctcaaatttcgggttggaacctccttgaccacgatgctttccaaaccaccctccttttttgcgcctgc
ctccatcaccctgaccccggggtccagtgcttgggccttctcctgggtcatctgcggggccctgctctatcgctcccgggggcacgtca
ggctcaccatctgggccaccttcttggtggtattcaaaataatcggcttcccctacagggtggaaaaatggccttctacctggagggggc
ctgcgcggtggagacccggatgatgatgactgactactgggactcctgggcctcttttctccacgtccacgacctctccccctggctcttt
cacgacttcccccccctggctctttcacgtcctctaccccggcggcctccactacctcctcgaccccggcctccactacctcctcgacccc
ggcctccactgcctcctcgaccccggcctccacctcctgctcctgcccctcctgctcctgcccctcctcctgctcctgcccctcctgcccc
tcctgctcctgcccctcctgcccctcctgctcctgcccctcctgcccctcctgctcctgcccctcctgcccctcctcctgctcctgcccctc

ctgcccctcctcctgctcctgcccctcctgcccctcctgctcctgcccctcctgcccctcctgctcctgcccctcctgcccctcctgctcct
gcccctcctgctcctgcccctcctgctcctgcccctcctgctcctgcccctcctgcccctcctgcccctcctcctgctcctgcccctcctgc
tcctgcccctcctgcccctcctgcccctcctgctcctgcccctcctcctgctcctgcccctcctgcccctcctgcccctcctcctgctcctg
ccctcctgcccctcctcctgctcctgcccctcctcctgctcctgcccctcctgcccctcctgcccctcctcctgctcctgcccctcctgcc
cctcctcctgctcctgcccctcctcctgctcctgcccctcctgcccctcctgcccctcctcctgctcctgcccctcctcctgctcctgcccct
cctgcccctcctgcccctcctgcccctcctcctgctcctgcccctcctcctgctcctgcccctcctgctcctgcccctcccgctcctgctcc
tgctcctgttccaccgtgggtccctttgcagccaatgcaacttggacgtttttggggtctccggacaccatctctatgtcttggccctgatcc
tgagccgcccggggctcctggtcttccgcctcctcgtcctcgtcctcttccccgtcctcgtccatggttatcacccccctcttctttgaggtcc
actgccgccggagccttctggtccagatgtgtctcccttctctcctaggccatttccaggtcctgtacctggcccctcgtcagacatgattc
acactaaaagagatcaatagacatctttattagacgacgctcagtgaatacagggagtgcagactcctgccccctccaacagccccccc
accctcatccccttcatggtcgctgtcagacagatccaggtctgaaaattccccatcctccgaaccatcctcgtcctcatcaccaattactc
gcagcccggaaaactcccgctgaacatcctcaagatttgcgtcctgagcctcaagccaggcctcaaattcctcgtcccccttttgctgg
acggtagggatggggattctcgggacccctcctcttcctcttcaaggtcaccagacagagatgctactggggcaacggaagaaaagct
gggtgcggcctgtgaggatcagcttatcgatgataagctgtcaaacatgagaattcttgaagacgaaagggcctcgtgatacgcctattt
ttataggttaatgtcatgataataatggtttcttagacgtcaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaa
atacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttc
cgtgtcgcccttattcccttttttgcggcattttgccttcctgtttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagt
tgggtgcacgagtgggttacatcgaactggatctcaacagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatga
gcacttttaaagttctgctatgtggcgcggtattatcccgtgttgacgccgggcaagagcaactcggtcgccgcatacactattctcagaa
tgacttggttgagtactcaccagtcacagaaaagcatcttacggatggcatgacagtaagagaattatgcagtgctgccataaccatgag
tgataacactgcggccaacttacttctgacaacgatcggaggaccgaaggagctaaccgcttttttgcacaacatgggggatcatgtaa
ctcgccttgatcgttgggaaccggagctgaatgaagccataccaaacgacgagcgtgacaccacgatgcctgcagcaatggcaacaa
cgttgcgcaaactattaactggcgaactacttactctagcttcccggcaacaattaatagactggatggaggcggataaagttgcaggac
cacttctgcgctcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcact
ggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgct
gagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatactttagattgatttaaaacttcatttttaatttaaa
aggatctaggtgaagatccttttgataatctcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaa
gatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgcc
ggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagtgtagccgtagtta
ggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcg
tgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggggttcgtgcacacagcccagc
ttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcgg
acaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcct
gtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcgg

ccttttttacggttcctggccttttgctggccttgaagctgtccctgatggtcgtcatctacctgcctggacagcatggcctgcaacgcgggc
atcccgatgccgccggaagcgagaagaatcataatggggaaggccatccagcctcgcgtcgcga

S5

SCORE SHEET and MONITORING- Paragraph 56.2.1

One score sheet represents one cage with the maximum of 5 animals. In case of obtaining the score result ≥6 in one of the animals, this particular animal will be entitled to its separate, individual score sheet.

I. Routine Monitoring will consist of:
   a) Checking presence of food and water, the bedding state, animals wellbeing – **performed daily in the first 3 days after injection and then 3 times per week (Form A filled out)**
   b) Visual Inspection (performed 3 times a week – SCORE SHEET filled out):
      - Examination from a distance (performed 3 times a week – SCORE SHEET filled out)
      - Observation after stimulus (performed 3 times a week – SCORE SHEET filled out)

   If any of the animals looks/behaves suspicious (all animals at least once a week or more if indicated by the score obtained)

      - Close observation of an individual animal after restrain (at least once a week up to daily depending on the scoring result - SCORE SHEET filled out)

					Scores				
Date	Time	Room No	Cage No	Animal ID	Gen. Appear., Mobility	Tumor size/Necr.	SUMMARY	Final Proceedings	Researcher

Descriptions and scoring:

1) **Gen. Appear., Mobility -** General Appearance (includes skin, orifices, posture and locomotion) – please see Appendix I at the end of this policy for graphics to be used for the BCS (Body Conditioning Scale).

Description	Score
No changes, normal behaviour and locomotion, fur clean and well groomed, pink mucus membranes, bright, alert, responsive (BCS = 3)	**0**
Fur not well groomed or excessive grooming, less active, normal posture and locomotion, normal respiratory rate 60-220/min	**2**
Skin or hair coat in poor condition (scruffy, dirty, fur loss), excessive licking and scratching of the affected site, less active or hyperactive, disoriented, aggressive, decreased signs of grooming, pale mucus membranes (BCS = 2), normal posture	**8**
Skin or coat in poor condition (swellings, atypical wrinkles, inflammatory reaction), self-damage, dehydration (decreased skin elasticity), emaciated, inactive, restriction of movement/motor activity, abnormal posture (curvature of the back, stretching of head and neck, up-drown abdominal wall, stretching of the body) (BCS = 1)	**12**
Moribund or severe cachexia, immobile	**12**

2) **Tumor size/Necr. –** Tumor size, degree of necrosis, tumor characteristics

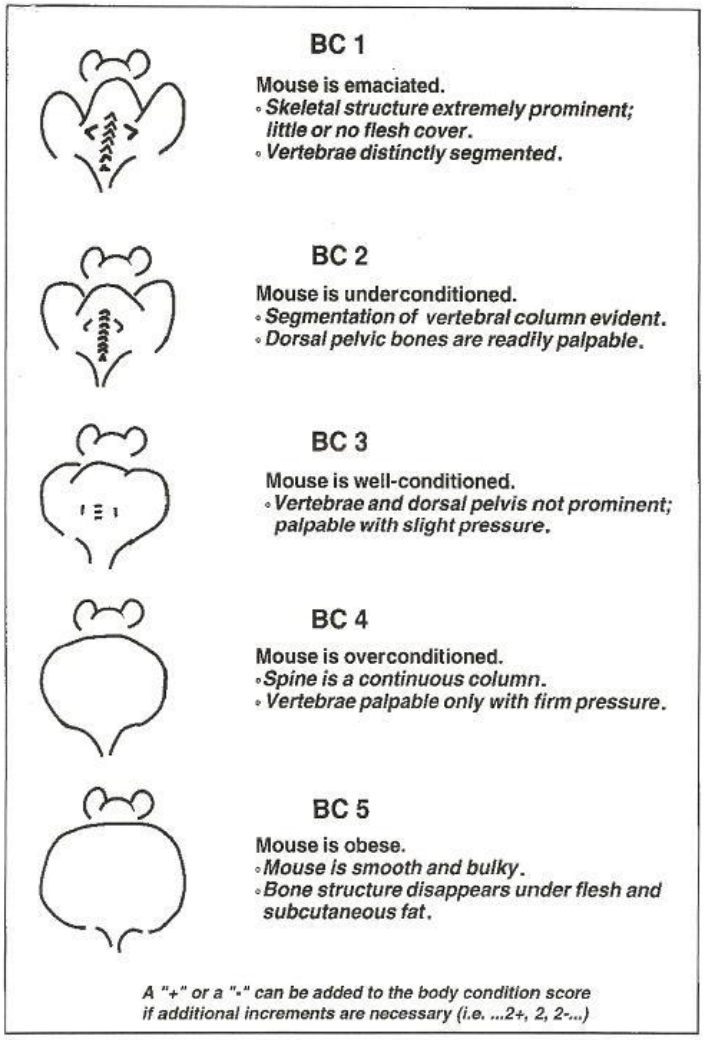
Description	Score
No tumor, tumor very small (≤ 5 mm length at its longest diameter), not limiting mobility, not limiting ability to eat or breathe	**0**
Recurrent scratching/biting of tumor, acute burst releasing fluid, tumor is pronounced, ≥ 5 mm length at its longest diameter	**4**
Locomotion impeded, chronically wet/weeping scab/crust or solid yellow matter exposed, ulceration, tumor size ≥ 15 mm length at its longest diameter	**12**
Bleeding, raw tissue exposed, white basal layer exposed, necrosis	**12**

Final Proceedings - Summation of scores and course of action

Action	Scores Summary
Routine monitoring	**0-5**
Close observation – up to daily monitoring. May put food and/or a water source on cage bottom and/or give SC or IP saline or Lactated Ringer's Solution.	**6-11**
Animals have to be euthanized.	≥**12**

S6

Body Condition Scoring (BCS)



Study Licence Number: 166/2014

Responsible Person: …….

Contact details: ……